Myasthenia Gravis: Unravelling the ATP role on neuromuscular transmission

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____

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With the end of this work, i end one of the best stages of my life.

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Abstract

Adenosine triphosphate (ATP) is co-released together with acetylcholine (ACh) upon electrical stimulation of motor nerve terminals (Magalhães-Cardoso *et al.*, 2003). Released ATP modulates neuromuscular transmission either by directly activating P2 purinoceptors (P2R) (Salgado *et al.,* 2000) or indirectly through the activation of P1 receptors after being metabolized into adenosine (ADO), via ecto-nucleotidases (Correia-de-Sá *et al.*, 1996). Adenosine derived from the extracellular catabolism of ATP activates preferentially excitatory A_{2A} receptors at stimulated motor nerve terminals (Correia-de-Sá *et al.,* 1996; Cunha *et al.,* 1996a). We have recently demonstrated that tonic activity of A_{2A}R is impaired in *Myasthenia gravis* probably due to low levels of endogenous ADO accumulation (Oliveira *et al.,* 2015a) indicating that neuromuscular transmission failure is associated to deficits in the ADO pathway.

Considering the crucial role of ATP on neuromuscular transmission mediated by P1 and P2 receptores we decided to evaluate the amounts of endogenous ATP release as well as extracellular catabolism of ATP at the rat neuromuscular junction from an experimental autoimmune *Myasthenia gravis* (EAMG) animal model. Moreover, ATP modulation of neurotransmitter release was also evaluated in health and EAMG model.

EAMG was induced in Wistar rats through immunization with R97-116 peptide, an immunogenic sequence of the α subunit of the rat nicotinic AChR (Oliveira *et al.,* 2015a). Control animals received the CFA emulsion without the peptide. Animals from the Naïve group were not submitted to treatment. Phrenic nerve hemidiaphragm preparations were isolated and the release of [^{3}H]ACh and ATP was evoked by phrenic nerve stimulation with 5 Hz-trains (750 pulses of 0.04 ms duration). ATP and [^{3}H]ACh content was measured by the luciferin-luciferase bioluminescence assay and by liquid scintillation spectrometry, respectively, and kinetics of the extracellular ATP catabolism by HPLC (UV Detetion).

EAMG animals exhibited an increased levels of electrically induced ATP release (0,139±0,039 pmol/mg, *n*=4) comparatively to CFA (0,051±0,006 pmol/mg, *n*=6) animals. The increased accumulation in the bath effluent of ATP is not related to a decrease in ATP catabolism at rat motor nerve terminals from EAMG rats. In fact, ATP (30 μM) catabolism was increased since the half-degradation time of ATP was

decreased in EAMG (5±1 min, *n*=4) animals comparatively to Naïve (8±2 min, *n*=8) rats. In parallel, an increased accumulation for the metabolite ADP, during the firsts 15 minutes of ATP metabolism, was quite evident in EAMG animals. The rat motor nerve terminals seems to be equipped with inhibitory P2R, since the slowly hydrolysable analogue of ATP, βγImidoATP (100 μM) inhibited (21±11%, *n*=3) neurotransmitter release. However, the presynaptic inhibitory P2 receptores are only operative in the absence of ADO since ATP (1 μM) only presented an inhibitory effect (1) in the presence of adenosine deaminase (ADA 0,5 U/mL), the enzyme that inactivates ADO into INO (32±18%, *n*=4), and when incubated for shorter periods (3 min) which impairs ADO accumulation from ATP catabolism. Interestingly, this results points to a possible crosstalk between presynaptic inhibitory P2R and facilitatory A_{2A}R. However, this hyphotesis needs to be clarified in our experimental conditions. Nevertheless it has been recently documented a similar interaction in other systems, like the bladder (Yu *et al.,* 2014). In the same way, the inhibitory effect of P2R was only observed at the rat motor nerve terminals from EAMG animals when ATP (1 μM) was applied with 3 minutes incubation (41±3%, *n*=2). Despite the fact we have not collected direct evidences for an higher susceptibility of presynaptic inhibitory P2R to desensitization in EAMG animals, we may speculate that this fenomema is operating in EAMG animals since (1) they exhibited higher amounts of evoked release ATP (2) the ATP metabolite ADP, a ligand for inhibitory P2R like $P2Y_{12}$ and $P2Y_{13}$ receptors, accumulates in higher concentrations at the synaptic cleft comparatively to naïve animals and (3) $P2Y_{12}$R have been reported to be desensite in other systems, like bladder and plaquets. In addition, application of ATP (1 μM) 15 min prior stimulus recovered A_{2A}R activity in EAMG animals since ATP (1 μM) facilitated [^{3}H]ACh release by (43±12%, *n*=5) and in the presence of ADA (0,5 U/mL), failed to affect transmitter (5±17%, *n*=4)

In conclusion, ATP modulates neurotransmitter release at rat motor nerve terminals from both healthy and EAMG animals by activating P_2R and A_{2A}R. In spite of that, activation of presynaptic facilitatory A_{2A}R activation prevails over inhibitory P2YR which may resuly from higher susceptibility to desensitization of P2YR probably due to a crosstalk with A_{2A}R.

Keywords: ATP, Neuromuscular transmission, *Myasthenia Gravis*, nAChR, experimental autoimmune *Myasthenia gravis* (EAMG).

Resumo

O ATP (adenosina trifostato) é libertado juntamente com a acetilcolina (ACh) mediante estimulação elétrica dos terminais nervosos motores (Magalhães-Cardoso *et al.*, 2003). Uma vez libertado, o ATP pode modular diretamente a transmissão neuromuscular, através da ativaçao de purinorecetores P2 (P2R) (Salgado *et al.,* 2000) ou indiretamente através da ativação de recetores P1 após ter sido metabolizado em adenosina (ADO) pela via das ecto-nucleotidases (Correia-de-Sá *et al.,* 1996). A adenosina resultante do catabolismo extracelular do ATP ativa preferencialmente os recetores excitatórios A_{2A} nos terminais nervosos motores (Correia-de-Sá *et al.,* 1996, Cunha *et al.,* 1996a). O nosso grupo demonstrou recentemente que a atividade tónica dos A_{2A}R encontra-se comprometida na *Myasthenia gravis*. Esta alteração está associada à diminuição nos níveis endógenos de ADO (Oliveira *et al.,* 2015a), sugerindo que o comprometimento da transmissão neuromuscular em EAMG poderá envolver alterações funcionais na via de conversão do ATP em ADO.

Considerando o papel crucial do ATP na transmissão neuromuscular mediado por recetores P1 e P2 decidimos avaliar os níveis endógenos de ATP, bem como o catabolismo extracelular do ATP na junção neuromuscular no modelo experimental auto-imune de *Miastenia gravis* (EAMG). Além disso, a modulação da libertação do ATP também foi avaliada em animais saudáveis e modelos EAMG.

O modelo animal EAMG foi induzido em ratazanas Wistar por meio de imunização com o péptido R97-116, uma sequência imunogénica da subunidade α do recetor nicotínico da acetilcolina (Oliveira *et al.,* 2015a). Os animais do grupo controlo receberam a emulsão de CFA sem o péptido. Os animais do grupo Naïve não foram submetidos ao tratamento. As preparações dos hemidiagramas dos nervos frénicos foram isoladas e a libertação de ACh e ATP foi induzida pela estimulação do nervo frénico com 750 pulsos (duração de 0,04 ms) aplicados com uma frequência de 5 Hz. A avaliação dos níveis endógenos de ATP e a libertação de [^{3}H]acetilcolina foram avaliadas pelo ensaio de bioluminescência luciferina-luciferase e por espectrometria de cintilação líquida, respectivamente. O catabolismo do ATP extracelular foi avaliado por HPLC (UV Detetion).

Nos animais EAMG observou-se um aumento nos níveis de libertação de ATP induzido por estimulação elétrica (0,139±0,039 pmol/mg, *n*=4) comparativamente com os animais CFA (0,051±0,006 pmol/mg, *n*=6 animais). O aumento dos níveis endógenos de ATP não está relacionado com uma diminuição do seu catabolismo nos terminais nervosos motores de ratazana EAMG, uma vez que o tempo de semi-vida do ATP foi menor no modelo EAMG (5±1 minutos, *n*=4), comparativamente com os animais Naïve (8±2 min, *n*=8). Em paralelo, observou-se um aumento da acumulação de ADP, durante os primeiros 15 minutos de metabolismo do ATP, nos animais EAMG. O análogo estável, βγImidoATP (100μM), inibiu (21±11%, *n*=3) a libertação do neurotransmissor, sugerindo que os terminais nervosos motores de ratazana possuem recetores P2 inibitórios. Estes recetores P2 inibitórios pré-sináticos exercem o seu efeito modulador apenas na ausência de ADO e consequente atividade dos $A_{2A}R$ dado que o ATP (1μM) apenas exibiu um efeito inibitório (1) na presença da desaminase da adenosine (ADA 0,5 U/mL), a enzima que inativa a ADO em INO (32±18%, *n*=4), e (2) quando incubado durante períodos mais curtos (3min) que envolvem a perda de acumulação de ADO a partir do catabolismo de ATP. Curiosamente, estes resultados apontam para uma possível interação entre os recetores pré-sináticos inibitórios P2R e $A_{2A}R$ facilitatórios. No entanto é necessário esclarecer esta hipótese nas nossas condições experimentais, até porque foi recentemente documentada uma interação semelhante em outros sistemas, como a bexiga (Yu *et al.,* 2014). Da mesma forma, o efeito inibitório de P2R na modulação da transmissão neuromuscular dos animais EAMG foi apenas observado quando o ATP (1μM) foi aplicado com 3 minutos de incubação (41±3%, *n*=2). Apesar de não se ter recolhido evidências diretas para uma maior susceptibilidade do P2R pré-sinático inibitório para o fenómeno de dessensibilização nos animais EAMG, podemos especular que este mecanismo está a ocorrer em condições de miastenia uma vez que o modelo EAMG (1) que exibiu um aumento dos níveis endógenos ATP, (2) o metabolito do ATP, ADP, um ligando para os P2R como $P2Y_{12}$ e $P2Y_{13}$, acumula-se em concentrações mais elevadas na fenda sinática e (3) os $P2Y_{12}R$ apresentam elevada susceptibilidade para a dessensibilização em outros sistemas, como tem sido referido em bexiga e plaquetas. Adicionalmente, a aplicação do ATP (1μM) 15 minutos antes do estímulo recupera a atividade dos $A_{2A}R$ em animais EAMG, uma vez que o ATP (1μM) facilitou a libertação de [^{3}H] ACh (43±12%, *n*=5) e na presença de ADA (0,5 U/ml), não conseguiu modificar a libertação do neurotransmissor (5±17%, *n*=4).

Em conclusão, ATP modula a libertação de neurotransmissores nos terminais nervosos motores de ambos os animais, saudáveis e EAMG, ativando P2R e

$A_{2A}R$. Apesar disso, a ativação pré-sináptica dos $A_{2A}R$ facilitatórios prevalece sobre a ativação P2YR inibitórios o que poderá ser devido a uma maior susceptibilidade para a dessensibilização dos P2YR provavelmente devido a uma interação com $A_{2A}R$.

Palavras-chave: ATP, Transmissão neuromuscular, *Miastenia gravis*, nAChR, modelo experimental auto-imune de *Miastenia gravis* (EAMG).

Index

List of figures and tables

Part of this work was presented at the following scientific meetings:

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Inhibition of histone deacetylases rehabilitates neuromuscular transmission in experimental autoimmune Myasthenia gravis

Oliveira L., Mota C., Fernandes M., Neves L., Correia-de-Sá P.

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Unravelling the role of ATP on neuromuscular transmission in an experimental model of autoimmune *Myasthenia gravis* (EAMG)

Timóteo M.A., Neves L., Fernandes M., Silva I., Correia-de-Sá P. and Oliveira L.

List of Abbreviations

A_1R - Adenosine A_1 receptor

$A_{2A}R$ - Adenosine A_{2A} receptor

A_3R - Adenosine A_3 receptor

Abs - Antibodies

ACh - Acetylcholine

AChE - Acetylcholinesterase

AChR - Acetylcholine receptor

ADA - Adenosine deaminase

ADO - Adenosine

ADP- Adenosine 5'-diphosphate

AMP - Adenosine 5'-monophosphate

ATP - Adenosine 5'-triphosphate

Ca^{2+} - Calcium ion

CD39 - E-NTPDase1

CD73 - Ecto-5'-nucleotidase

cAMP - Cyclic adenosine monophosphate

CFA - Complete Freund's adjuvant

DPM - Disintegrations per minute

EAMG - Experimental Autoimmune Myasthenia Gravis

EOM - Extraocular muscles

EPP - Endplate potential

HPLC - High pressure liquid chromatography

HX - Hipoxantine

[^{3}H]ACh - Tritiated Acetylcholine

IFA - Incomplete Freund's adjuvant

Ig - Immunoglobulins

IMP - Inosine monophosphate

INO - Inosine

IVIg - Intravenous immunoglobulins

M_1R - Muscarinic M_1 receptor

M_2R - Muscarinic M_2 receptor

MHC II - Major histocompatibility complex class II

mM - MiliMolar

mm - Millimeters

min - Minute

mAU - Milli Absorvance Units

mepp - Miniature endplate potential

mL - Milliliter

mg/Kg - Milligram per kilogram

***n* -** Sampling

Na^+ - Sodium ion

nAChR - Nicotinic acetylcholine receptor

NMJ - Neuromuscular junction

nM/mg - Nanomol per Milligram

NMT - Neuromuscular transmission

mA - Milliamps

MG - *Myasthenia Gravis*

MuSK - Muscle-specific kinase

PBS - Phosphate Buffered Saline

Pm - PicoMolar

P/Q - Type - Presynaptic $Ca_v2.1$ voltage-gated calcium channels

PLC - Phospholipase C

R97-116 - Syntetic peptide corresponding to region 97-116 of the rat nAChR α subunit

RNS - Repetitive nerve stimulation

SNARE - N-ethylmaleimide sensitive factor attachment receptor complex

SEM - Mean standard error

SFEMG - Single-fiber electromyography

SPF - Sociedade Portuguesa de Farmacologia

SVs - Synaptic vesicles

$TCD4^+$ - Effector T cells

U/mL - Unit per milliliter

UP - Universidade do Porto

µCi - MicroCurie

µM - MicroMolar

1. Introduction

1.1. *Myasthenia Gravis* - Pathophysiology

Myasthenia gravis (MG) is a B-cell-mediated, T-cell-dependent autoimmune disease characterized by excessive muscle weakness and fatigue (Zuckerman *et al*., 2010). The target of the autoimmune attack in most cases is the skeletal muscle acetylcholine receptor (AChR), but in others non-AChR components of the neuromuscular junction, such as the muscle-specific receptor tyrosine kinase may also be targeted (reviewed by Juel and Massey, 2007). These antibodies reduce the number of effective receptors to nearly one-third of the normal (Lindstrom, 2000) leading to a decrease in the safety margin of the neuromuscular transmission, which is particularly relevant during high-frequency nerve activity. Furthermore, the typical deep junctional folds are replaced by a relatively flat surface. The breakdown of self-tolerance in the thymus apparently leads to the development of anti-AChR autoantibodies (Baggi *et al.,* 2012; Melms *et al.,* 2006; Newsom-Davis *et al.,* 1981) with induction or activation of AChR-specific $CD4^+$ T helper cells and production of pro-inflammatory cytokines, consequently leading to the synthesis of high-affinity antibodies (Hoedemaekers *et al.,* 1997; Vincent *et al.,* 2003). Therefore, T cells play a pivotal role in MG since they lead the attack to the endplates by recognition of the antigen coupled to the major histocompatibility complex (MHC) class II molecules, promoting B cell production of anti-AChR antibodies by plasmocytes (Aricha *et al.*, 2006; reviewed by Juel and Massey, 2007; Vincent *et al.*, 2003).

1.1.2. Diagnostic

MG remains one of the most challenging medical diagnoses due to its fluctuating character and to the similarity of its symptoms to those of other disorders (reviewed by Juel and Massey, 2007). Although a formal clinical classification system and research standards have been established for MG, there are no generally accepted formal diagnostic criteria. The most essential elements of diagnosis are clinical history and examination findings of fluctuating and fatigable weakness, mostly involving extraocular and bulbar muscles. A clinical diagnosis may be confirmed by laboratory testing including a pharmacologic testing with edrophonium chloride (Tensilon test), an acetylcholinesterase inhibitor, that elicits unequivocal improvement in strength; an electrophysiologic testing, with repetitive nerve stimulation (RNS) studies and/or single-fiber electromyography (SFEMG) that demonstrates a primary postsynaptic

neuromuscular junctional disorder; or by serological demonstration of AChR or MuSK antibodies (reviewed by Juel and Massey, 2007).

1.1.3. Symptoms

MG is characterized by varying degrees of weakness to the muscles of the body, and most commonly affects the muscles that control the eyes, facial expressions, chewing, talking and swallowing. The disease can also affect muscles associated with breathing, neck and limb movement (Téllez-Zenten *et al.,* 2004). Approximately 50 percent of people with MG present with ocular symptoms of ptosis and/or diplopia. About 15 percent present with bulbar symptoms, which include dysarthria, dysphagia and fatigable chewing. Fewer than 5 percent present with proximal limb weakness alone (Bird *et al.,* 2014). A myasthenic crisis occurs when muscles that control breathing weaken to the point that ventilation is inadequate, creating a medical emergency and requiring assisted ventilation. In individuals whose respiratory muscles are affected by their disease, a crisis may be triggered by infection, fever, surgery, emotional stress or an adverse reaction to medication (Téllez-Zenten *et al.,* 2004).

1.1.4. Epidemiology

Although MG is rare, prevalence rates for MG have increased over time, possible due to improvements in diagnosis. Recent prevalence rates approach 20/100 000. The onset of MG is influenced by gender and age in a bimodal fashion. In patients younger than 40, women predominate with a ratio of 7:3. In the fifth decade, new cases of MG are evenly distributed between men and women. After age 50, new cases of MG are slightly more common in men with a ratio of 3:2 (reviewed by Juel and Massey, 2007).

1.1.5. Therapeutic

Treatment of MG is based on four different options which take different amounts of time before muscular weakness will improve (Fig. 1):

- Improvement of neuromuscular transmission by acetylcholinesterase inhibitors, e.g. pyridostigmine, neostigmine and physostigmine;

- Treatment of acute exacerbations (plasmapheresis, immunoadsorption, intravenous immunoglobulin);

- Immunosuppression, e.g. corticosteroids, azathioprine and monoclonal antibodies

- Thymectomy.

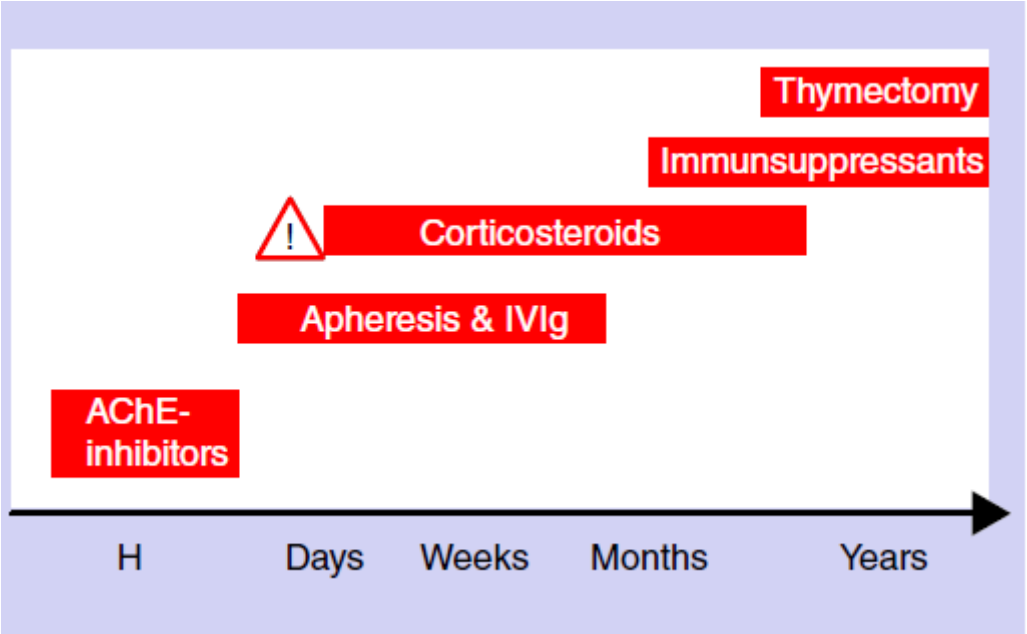

Fig. 1 - Onset of action of the different therapy options in myasthenia (adapted from Sieb, 2014).

Therapy usually begins with acetylcholinesterase inhibitors. Acetylcholinesterase inhibitors slow the hydrolysis of ACh at the neuromuscular junction and provide temporary improvement in strength in many patients with MG but do not retard the underlying autoimmune attack on the neuromuscular junction. Corticosteroids are the most widely used immune modulating agents for MG. Although the mechanism of action in MG is unknown, corticosteroids have numerous effects on the immune system including reduction of cytokine production. Azathioprine is an effective agent for long-term immune modulation in MG as a steroid sparing drug or as initial immunotherapy. Compared to corticosteroids, azathioprine has a favorable side effect profile for long term use (reviewed by Juel and Massey, 2007). In some situations, plasma exchange can be used in MG to achieve rapid, temporary improvement in strength. During plasma exchange, plasma containing nAChR antibodies is separated from whole blood and replaced by albumin or fresh frozen plasma. Also, intravenous immunoglobulins (IVIg) can be used to bind the circulating antibodies. Thymectomy has also been widely performed in an effort to achieve medication-free remission in MG. However, and because of the increased surgical risk and reduced life span, thymectomy is rarely performed in MG patients nowadays. For each patient, an individual treatment plan

must be compiled that will be adjusted further on the therapeutic response during the course. Using the spectrum of treatment options available nowadays, the majority of myasthenic patients can have largely normal lives. Since these therapies have quite short-term benefits (reviewed by Juel and Massey, 2007), it is critical to find new therapeutic strategies with less side effects. The overall goal is to reestablish normal clinical neuromuscular function while minimizing adverse side effects.

1.2. Animal models to study *Myasthenia gravis*

Animal models allow a better understanding of the pathophysiological processes of human diseases. It is important to have in mind the similarities and the differences of the species chosen, and the resemblance pathology and outcome of an induced disease or disorder in the model species, with the respective lesions of the target species, so the experimental results can be extrapolated from one species to the other (Hau and Van Hoosier, 2003). Few induced models completely mimic the target disease in Human's (Hau and Van Hoosier, 2003). Patrick and Lindstrom in 1973 immunized rabbits in order to obtain autoantibodies against the recently purified AChR, and observed that the animals developed weakness and electrophysiological abnormalities that were similar to those in human MG (Patrick and Lindstrom 1973). This experimental disorder in rabbits, received lately the name Experimental Autoimmune *Myasthenia Gravis* (EAMG). Later EAMG was reproduced in other species (Lennon *et al.,* 1975) and has contributed with a great deal of information for unveiling the molecular and immunological features of this disease. There are numerous procedures to create an animal model for MG. A very common one, which recreates most of the observed symptoms of MG in Humans, consists on injecting rodents with anti-nAChR Abs and/or their immunization with nAChRs isolated from *Torpedo californica* (Aricha *et al.,* 2006). Baggie and collaborators showed that the breaking of tolerance to a single T cell epitope of the self-autoantigen induces autoreactive T cells and specific Abs to rat AChR (Baggi *et al.,* 2004). This model was established by immunizing a susceptible rat strains (*Lewis* rats) with a synthetic peptide corresponding to region α97-116 of the rat AChR α subunit, in CFA (Complete Freund's Adjuvant – a mixture of oils and water plus killed *Mycobacterium tuberculosis* strain, used to stimulate immune response). This model of EAMG is valid as a model to understand the key immunological processes and molecular aspects, leading to MG as well as providing a practical instrument for testing the capability of possible treatment methods for MG and other antibody-mediated autoimmune diseases (Baggi *et al.,* 2012).

However, experimental MG differs from human disease in a few features (Table 1).

Despite, myasthenic patients commonly present thymic alterations, suggesting a potential role of the thymus in the pathogenesis of the disease (Meinl *et al.,* 1991), induced animals develop EAMG after AChR-immunization and the auto-sensitization process seems to occur only in draining lymph nodes (Christadoss *et al.,* 2000), apparently without affecting the thymus, as in MG patients.

Similarities	Differences
Immunopathological features	
Presence of anti-AChR Abs in the serum; Deposits of IgGs and C3 complement component at the NMJ; Loss of muscle nAChRs; MHC class II-restricted presentation of AChR epitopes; Involvement of T helper cells in B - cell antibody production.	Disease does not arise spontaneously in animals, needs for induction factors; Involvement of the thymus (present in some cases of MG, absent in EAMG). Thymic alterations are absent in EAMG, and in MG patients, hypertrophy and thymomas are often present; Phagocytic cells detected in the acute phase of rat EAMG, are absent at the NMJ of human MG patients.
Clinical manifestations	
Muscle weakness, most prominent in the upper body; Decreased response in the repetitive nerve stimulation test; Reduction in the miniature end-plate potential amplitude; Temporary improvement in muscle strength after anti-AChE treatment (Tensilon test).	Absence of ocular signs; Absence of relapse and remission periods.

Table 1 - Similarities and differences between MG and EAMG (Adapted from Baggi *et al.*, 2012).

1.3. The neuromuscular junction: Structure and function

The terminal arborization of α–motor neuron axons from the ventral horns of the spinal cord and brainstem provides the nerve terminals that form the NMJ (Fig. 2). These myelinated axons reach the muscles through peripheral nerves; then each axon divides into branches that innervate many individual muscle fibers (Conti-Fine *et al.,* 2006). Neuromuscular transmission at the skeletal muscle occurs when a quantum of ACh from the nerve ending is released and binds to the nAChRs on the postjunctional muscle membrane. The nerve terminal contains synaptic vesicles (SVs), each of which contains 5000–10 000 molecules of ACh (Hirsch, 2007). The content of a single vesicle is referred to as a 'quantum' of the transmitter. Occasional spontaneous release of quanta of ACh results in the production of a so-called miniature endplate potential (mepp) at the postsynaptic membrane. The arrival of the action potential at the nerve terminal results in opening of the voltage-gated calcium (P/Q and possibly N-type) channels (Hirsch, 2007), which are arranged in regular parallel arrays at the active zones. When the nerve action potential reaches the synaptic boutons, the depolarization opens voltage-gated Ca^{2+} channels on the presynaptic membrane. This Ca^{2+} influx triggers fusion of synaptic vesicles with the presynaptic membrane and ACh release. The ACh diffuses into the synaptic cleft where it can be hydrolyzed by AChE or binding to nAChR. The binding of ACh to postsynaptic nAChR thereby triggers the influx of Na^{+} through nAChR channel pore into the muscle fiber. The resulting endplate potential (EPP) activates voltage-gated Na^{+} channels, leading to further influx of Na^{+} and spreading of the action potential along the muscle fiber (Conti-Fine *et al.,* 2006).

The postsynaptic transmembrane protein, muscle-specific tyrosine kinase (MuSK) (Fig. 2), can be also an autoantigen in some MG patients (Hoch *et al.,* 2001). MuSK expression in both developing and mature muscle is similar to that of nAChR. In mature muscle, MuSK is present prominently only at the NMJ, where it is part of the receptor for agrin. Agrin is a protein synthesized by motor neurons and secreted into the synaptic basal lamina. The signaling mediated by agrin/MuSK interaction triggers and maintains rapsyn-dependent clustering of nAChR and other postsynaptic proteins (Ruegg and Bixby, 1998). Rapsyn, a peripheral membrane protein exposed on the cytoplasmic surface of the postsynaptic membrane, is necessary for clustering of nAChR, with which it coclusters. Rapsyn and AChR are present in equimolar concentrations at the NMJ, and they may be physically associated. Rapsyn causes clustering of NMJ proteins other than the nAChR, including MuSK. Mice lacking agrin or MuSK fail to form NMJs and die at birth of profound muscle weakness, and their

AChR and other synaptic proteins are uniformly expressed along the muscle fibers (Glass *et al.,* 1996).

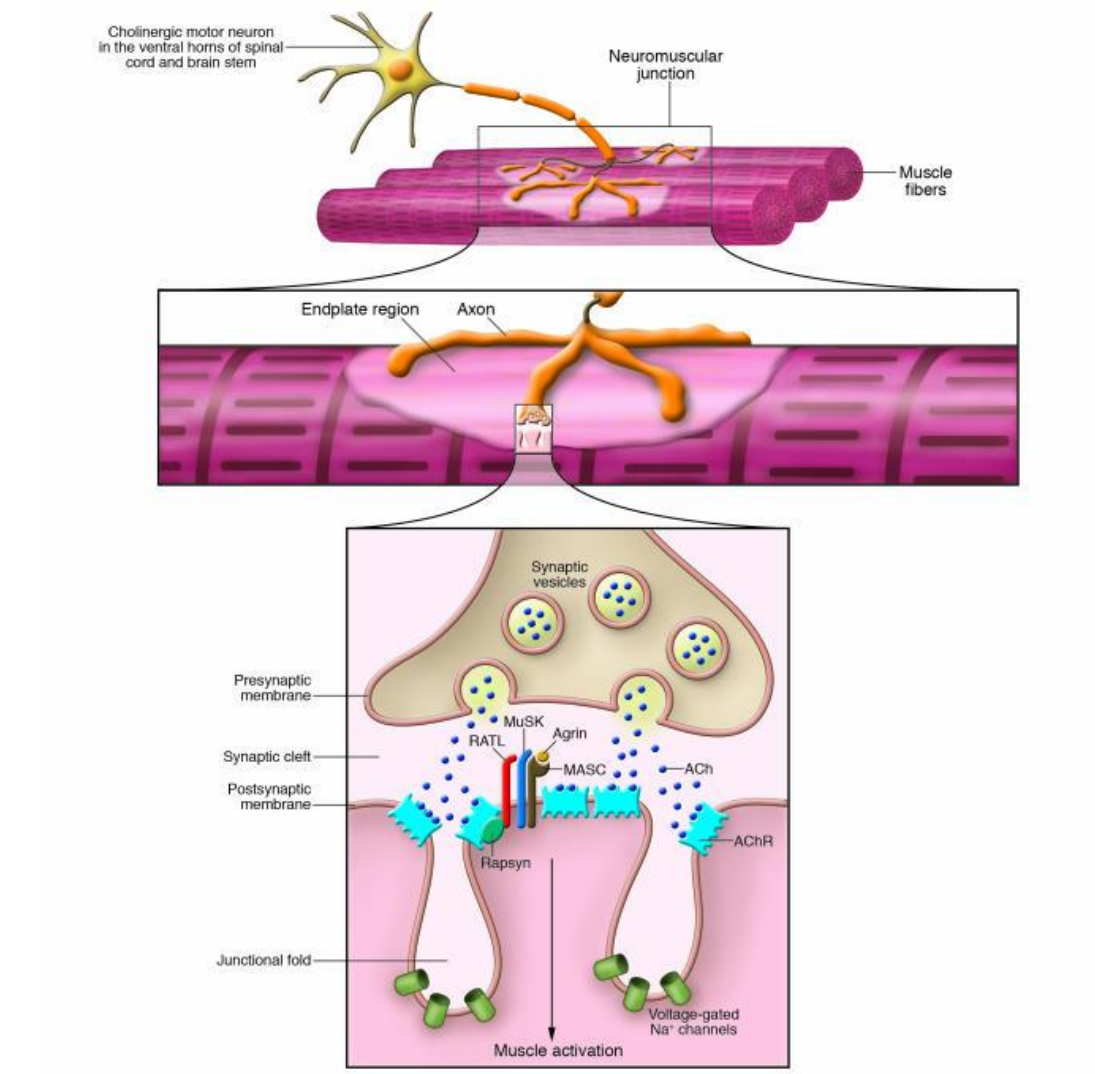

Fig. 2 - Structure of the NMJ (adapted from Conti-Fine *et al.,* 2006).

1.3.1. NMJ properties that influence susceptibility to muscle weakness in MG

The EPP generated in normal NMJs is larger than the threshold needed to generate an action potential. This difference may vary in different muscles, as discussed below. Neuromuscular transmission safety factor is defined as the ratio between the actual EPP and the threshold potential required to generate the muscle action potential. Its reduction is the electrophysiological defect that causes MG symptoms (Conti-Fine *et al.,* 2006). Also, the postsynaptic folds (Fig. 2) form a high-resistance pathway that focuses endplate current flow on voltage-gated Na^+ channels

in the depths of the folds, thereby enhancing the safety factor. A reduction in the number or activity of the nAChR at the NMJ decreases the EPP, which may still be adequate at rest. However, when the quantal release of ACh is reduced after repetitive activity, the EPP may fall below the threshold needed to trigger the action potential (Conti-Fine *et al.,* 2006). NMJ properties vary among muscles and may influence muscle susceptibility to MG (Hughes *et al.,* 2006). This is well illustrated by the NMJ of the extraocular muscles (EOMs), which are especially susceptible to developing myasthenic weakness. The NMJs of EOMs differ from those of skeletal muscle in several ways. They have less prominent synaptic folds, and therefore fewer postsynaptic nAChRs and Na^+ channels, and a reduced safety factor (Khanna and Porter, 2002). They are subject to very high neuronal firing frequency, making them prone to fatigue. Also, they express less intrinsic complement regulators, making them more susceptible to complement-mediated injury (Kaminski *et al.,* 2004). In skeletal muscles, fast twitch fibers have NMJs with greater quantal contents, a greater degree of postsynaptic folding (Wood and Slater, 1997), and higher postsynaptic sensitivity to ACh than slow-twitch NMJs (Sterz *et al.,* 1983), and they have increased Na^+ current in the NMJ region (Ruff, 1996). These properties may make fast-twitch skeletal muscle fibers less susceptible to myasthenic failure than slow-twitch fibers.

1.4. Purinergic receptor subtypes

The potent actions of extracellular ATP on many different cell types implicates the action of membrane receptors (Burnstock, 2007). Purinergic receptors were first defined in 1976 (Burnstock, 1976), and 2 years later a basis for distinguishing two types of purinoceptor, identified as P1 and P2 (for adenosine and ATP/ADP, respectively), was proposed (Burnstock**,** 1978). Later the P2 receptors were subdivided into P2X (ionotropic) and P2Y (metabotropic) subtypes on the basis of its pharmacological profile (Burnstock and Kennedy, 1985; Abbracchio *et al.,* 2006; Abbracchio *et al.,* 2009; Burnstock, 1976).

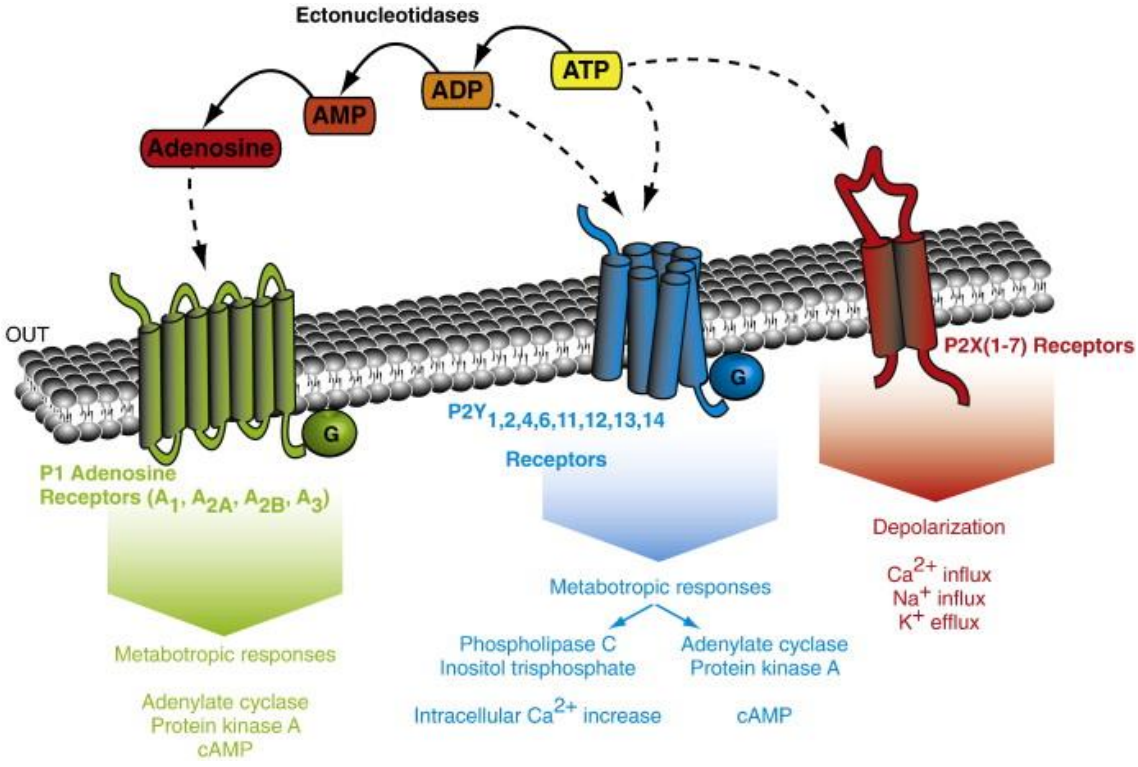

Fig. 3 - The purinergic receptor family. Extracellular ATP is the agonist of both P2X and P2Y receptors and is also the substrate of ectonucleotidases, which degrade ATP to adenosine and transiently generate ADP, providing the agonist for P2Y receptors. Adenosine, the final product of adenine nucleotide hydrolysis, activates P1 or adenosine receptors (Adapted from Baroja-Mazo *et al.*, 2013).

1.5. ATP on neuromuscular transmission

Over 30 years ago, it was demonstrated that adenosine 5′-triphosphate (ATP) is released from the motor nerve endings in the neuromuscular junction along with the major transmitter acetylcholine (ACh) (Silinsky, 1975). Released ATP modulates neuromuscular transmission either by directly activating P2 purinoceptors (P2R) (Salgado *et al.,* 2000) or indirectly through the activation of P1 receptors after being metabolized into adenosine (ADO), via ecto-nucleotidases pathway (Cunha *et al.*, 1996a).

1.5.1. Ectonucleotidases

Endogenous purine nucleotides (ATP, ADP, and AMP) released into the extracellular space may be converted ultimately to adenosine through a variety of cell surface-located enzymes referred to as ectonucleotidases. Within the past decade, ectonucleotidases belonging to several enzyme families have been discovered, cloned

and characterized. There are four major families of ectonucleotidases, namely the ectonucleotide triphosphate diphosphohydrolases (E-NTPDase, hydrolysing extracellular nucleoside tri- and diphosphates, but not monophosphates), comprising four surface-located different members (E-NTPDase 1,2,3 and 8), among which the ENTPDase1 is also called CD39; the ectonucleotide pyrophosphatase/ phosphodiesterase (E-NPP) family that can hydrolyse pyrophosphate 5'-monodiester bonds in ATP and dinucleoside polyphosphates or artificial substrates, comprising three different members (E-NPP1,2,3), among which the E-NPP3 is also called CD203; the ecto-5'-nucleotidase also called CD73, which hydrolyses only nucleosides monophosphates; finally, the alkaline phosphatases (AP) non-specific phosphomonoesterases, comprising five isoforms in the mouse, only one of which is expressed in the mammalian brain, which release inorganic phosphate from nucleoside 5'-tri-, 5'-di- and 5'-monophosphates (Zimmerman, 1996; reviewed by Noji *et al.,* 2004). The role of ectonucleotidases in synaptic transmission may be functionally relevant. They inactivate the released nucleotides and, thus, limit its temporal and spatial action. In situ the activity of ectonucleotidases is controlled by the substrate availability (Robson *et al.,* 2006). CD39 and CD73 are two major ectoenzymes that sequentially hydrolyze adenine nucleotides, leading to adenosine generation. CD39 hydrolyzes both ATP and ADP to AMP, which is subsequently converted to adenosine through CD73, a glycosyl phosphatidylinositol (GPI)-anchored molecule (Zimmermann, 1992). The ectonucleotidase pathway is regulated at the limiting step catalyzed by the CD73. This enzyme is subjected to feed-forward inhibition by ATP and/or ADP (Magalhães-Cardoso *et al.,* 2003). The extracellular ATP hydrolysis at NMJs shares many similarities with that described in many different preparations, being mainly catalysed by ectonucleotidases to form adenosine (Cunha, 2001). The main difference found in NMJs is the presence of an ecto-AMP deaminase activity that converts AMP into IMP (Cunha and Sebastião, 1991; Magalhães-Cardoso *et al.,* 2003). Indeed skeletal muscles fibers are one of the mammalian tissues with higher activity of AMP deamination (Ogasawara *et al.,* 1974; Magalhães-Cardoso *et al.,* 2003). Most of this enzymatic activity is associated with muscle fibers, but it has been shown that AMP deaminase is also present in the vicinity of neuronal and circulatory elements (Thompson *et al.,* 1992). Magalhães-Cardoso and colleagues (2003) have presented evidences demonstrating that ecto-AMP deaminase blunts the ATP-derived adenosine A_{2A} receptor facilitation of ACh release at rat motor nerve endings (Correia-de-Sá *et al.,* 1991; reviewed by Ribeiro *et al.,* 1996).

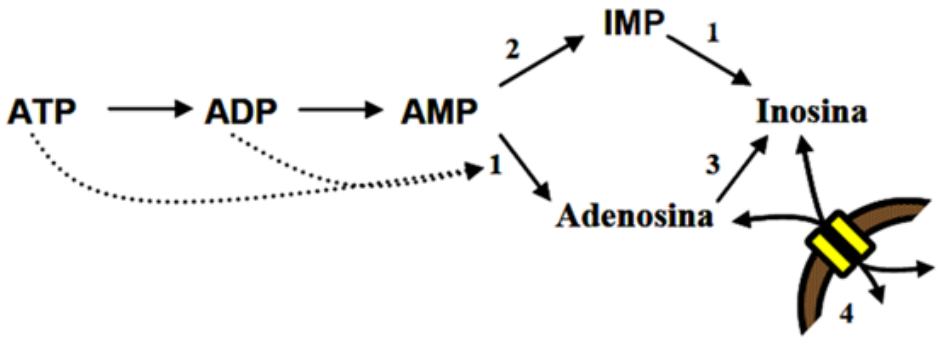

Fig. 4 - Extracellular catabolism of adenine nucleotides and nucleosides at the rat motor nerve terminals. The numbers in the figure represent: 1- ecto-5′-nucleotidase; 2- ecto-AMP deaminase; 3- ecto-adenosine deaminase; 4- adenosine transporter (adapted from Magalhães-Cardoso *et al.*, 2003).

1.5.2. ATP receptors activation – P2 receptors

Adenosine triphosphate (ATP) accumulates in the synaptic cleft of the neuromuscular junction (NMJ) during synaptic transmission due to the release of approximately equal quantities (Santos *et al.,* 2003) of ATP from nerve terminals (Cunha and Sebastião, 1991; Silinsky *et al.,*1999; Vizi *et al.,* 2000) and stimulated muscle fibres (Vizi *et al.,* 2000). This ATP does not appear to affect muscle fibres (Henning, 1997) but does influence the release of ACh (and co-released ATP) at the NMJ (Ribeiro and Walker, 1975). However, both enhancement (Salgado *et al.,* 2000) and reduction (Silinsky *et al.,* 1999) of neurotransmitter release by ATP have been reported. A possible explanation for the contrasting effects of ATP on neurotransmitter release at the NMJ is that it may act directly on ionotropic P2X receptors and/or metabotropic P2Y receptors, or indirectly via its metabolite adenosine on other metabotropic receptors (Ralevic and Burnstock, 1998). P2 receptors are activated by purines and some subtypes also by pyrimidines. Extracellular nucleotides act through purinergic receptors that comprise seven distinct P2X receptor subtypes ($P2X_{1–7}$), which act as ion channels, and eight P2Y receptor subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11–14}$) that are G protein coupled receptors (GPCRs) (Ralevic and Burnstock,1998).

Members of the existing family of ligand-gated nonselective cation channel $P2X_{1–7}$ receptor subunits show a subunit topology of intracellular -NH_2 and -COOH termini possessing consensus binding motifs for protein kinases; two transmembrane-spanning regions (TM1 and TM2). The TM1 is involved in the channel gating properties and the TM2 is associated to the ion pore. A large extracellular loop, with 10

conserved cysteine residues forming a series of disulfide bridges; hydrophobic H5 regions close to the pore vestibule, for possible receptor/channel modulation by cations (magnesium, calcium, zinc, copper, and proton ions); and an ATP-binding site, which may involve regions of the extracellular loop adjacent to TM1 and TM2 (Fig. 5B). The $P2X_{1–7}$ receptors show 30–50% sequence identity at the peptide level. The stoichiometry of $P2X_{1–7}$ receptor subunits is thought to involve three subunits that form a stretched trimer (Khakh *et al.,* 2001). It has become apparent that the pharmacology of the recombinant P2X receptor subtypes expressed in oocytes or other cell types is often different from the pharmacology of P2X receptor-mediated responses in naturally occurring sites. This is partly because heteromultimers as well as homomultimers are involved in forming the trimer ion pores. Spliced variants of P2X receptor subtypes might play a part (Chen *et al.,* 2000). For example, a splice variant of the $P2X_4$ receptor, while it is nonfunctional on its own, can potentiate the actions of ATP through the full-length $P2X_4$ receptors (Townsend-Nicholson *et al.,* 1999). Third, the presence in tissues of powerful ectoenzymes that rapidly break down purines and pyrimidines is not a factor when examining recombinant receptors, but is *in vivo*. $P2X_7$ receptors are predominantly localized on immune cells and glia, where they mediate proinflammatory cytokine release, cell proliferation, and apoptosis. $P2X_7$ receptors, in addition to small cation channels, upon prolonged exposure to high concentrations of agonist, large channels, or pores are activated that allow the passage of larger molecular weight molecules. The possible mechanisms underlying the transition from small to large channels have been considered (Egan *et al.,* 2006). The P2X receptor family shows many pharmacological and operational differences (Gever *et al.,* 2006). The kinetics of activation, inactivation, and deactivation also vary considerably among P2X receptors. The best characterized mechanism underlying the activity of these receptors result from their high permeability for Ca^{2+}. Activation of P2X receptors induces an increase in intracellular Ca^{2+} and depolarization wave, which leads to signal transmission. Functional interactions with other ion channels, K^+ outflow and Na^+ influx are also involved in P2X receptors signaling (Khakh and North, 2012).

$P2X_7$ was the first P2 receptor to be identified at the neuromuscular junction, although ATP and its metabolites ADP and adenosine have long been known to affect synaptic transmission at the NMJ (Fu and Poo, 1991). $P2X_7$ receptor subunits are present at all NMJs in a wide variety of skeletal muscles studied from birth into adulthood (Moores *et al.,* 2005).

Metabotropic P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$) are characterized by a subunit topology of an extracellular -NH_2 terminus and intracellular -COOH terminus, the latter possessing consensus binding motifs for protein kinases; seven transmembrane-spanning regions, which help to form the ligand-docking pocket; a high level of sequence homology between some transmembrane-spanning regions, particularly TM3, TM6, and TM7; and a structural diversity of intracellular loops and COOH terminus among P2Y subtypes, so influencing the degree of coupling with Gq/11, Gs, Gi, and Gi/o proteins (Abbracchio *et al.,* 2006) (Fig. 5C). Each P2Y receptor binds to a single heterotrimeric G protein (Gq/11 for $P2Y_{1,2,4,6}$), although $P2Y_{11}$ can couple to both Gq/11, and Gs, whereas $P2Y_{12}$ and $P2Y_{13}$ couple to Gi and $P2Y_{14}$ to Gi/o. Many cells express multiple P2Y subtypes (Abbracchio *et al.,* 2006 and Volonté *et al.,* 2006). P2Y receptors show a low level of sequence homology at the peptide level (19–55% identical) and, consequently, show significant differences in their pharmacological and operational profiles. Some P2Y receptors are activated principally by nucleoside diphosphates ($P2Y_{1,6,12}$), while others are activated mainly by nucleoside triphosphates ($P2Y_{2,4}$). Some P2Y receptors are activated by both purine and pyrimidine nucleotides ($P2Y_{2,4,6}$), and others by purine nucleotides alone ($P2Y_{1,11,12}$). In response to nucleotide activation, recombinant P2Y receptors either activate phospholipase C (PLC) and release intracellular calcium or affect adenylyl cyclase and alter cAMP levels (Burnstock, 2007).

Recent evidence suggests that ATP can play a role in forming and maintaining the postsynaptic specializations by activating its corresponding receptors (Tsim and Barnard, 2002).

Several lines of evidence support the co-existence and co-activity of $P2Y_1$ and $P2Y_2$ receptors at the NMJS (Choi *et al.,* 2001). The co-localization of these two P2Y receptors at the NMJ has now been observed in the four diverse species so far examined, i.e., mouse, rat, a bird, and an amphibian (Choi *et al.,* 2001), suggesting that it has a distinct significance in muscle function.

De Lorenzo and colleagues (2006), demonstrated that, at the mouse neuromuscular junction, extracellular ATP induced presynaptic inhibition of spontaneous ACh release via activation of P2Y receptors.

Recently, Giniatullin and colleagues (2015), revealed key steps in the purinergic control of synaptic transmission via $P2Y_{12}$ receptors associated with lipid rafts, and

identified NADPH oxidase as the main source of ATP-induced inhibitory ROS at the neuromuscular junction.

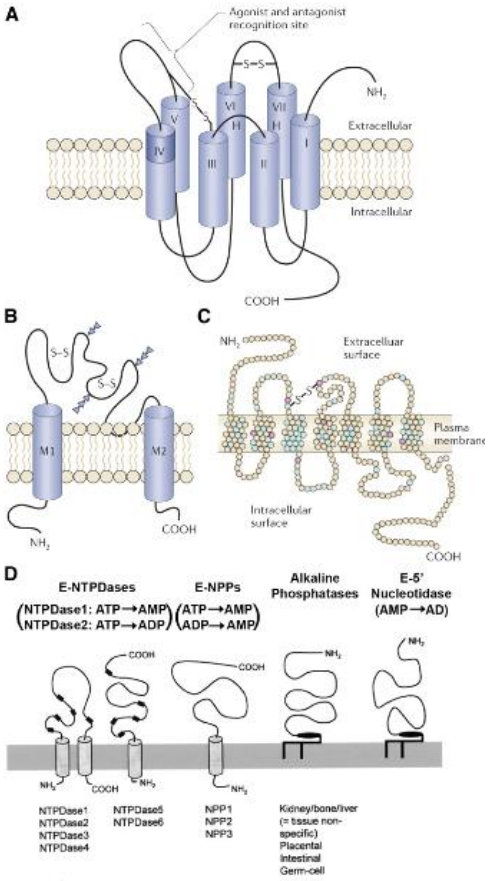

Fig. 5 - Membrane receptors for extracellular adenosine and ATP. A- The P1 family of receptors for extracellular adenosine are G protein-coupled receptors (S-S; disulfide bond). B- The P2X family of receptors are ligand-gated ion channels (S-S; disulfide bond; M1 and M2, transmembrane domains). C- The P2Y family of receptors are G protein-coupled receptors (S-S; disulfide bond; green circles represent amino acid residues that are conserved between $P2Y_1$, $P2Y_2$, and $P2Y_6$ receptors; fawn circles represent residues that are not conserved; and red circles represent residues that are known to be functionally important in other G protein-coupled receptors). D- Predicted membrane topography of ectonucleotidases, consisting of the ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) family, the E-NPP family, alkaline phosphatases, and ecto-5′-nucleotidase (adapted from Burnstock, 2007).

2. Adenosine as a neuromodulator

Adenosine (ADO) is a ubiquitous molecule and an essential component of all living cells. This nucleoside is involved in key processes of the primary metabolism, especially the metabolism of nucleotides, nucleosides and amino acids that have sulfide groups and in the modulation of cellular metabolic state (e.g. transmethylation reactions and ammonia processing) (Cunha, 2001; Cunha, 2005; Stone, 1985). The first description that suggests that ADO and its precursor, adenosine triphosphate (ATP) could affect neuronal function has been advanced by Drury and Szent-Gyorgyi (1929). Later studies in the neuromuscular junction (Ginsborg and Hirst, 1972; Ribeiro and Walker, 1973) and cortical neurons (Phillis *et al.,* 1974) have shown that actually ADO plays a neuromodulatory role. ATP is stored in synaptic vesicles and can also be released by nerve terminals during depolarization (Zimmermann, 1994). Previous studies using NMJs from different species reported that nerve stimulation triggers the release of ATP from the motor nerve terminal to the synaptic cleft (Magalhães-Cardoso *et al.,* 2003; Santos *et al.,* 2003). Most commonly, ATP co-released with ACh from motor nerve terminals is metabolized extracellularly via the ecto-nucleotidase pathway that sequentially catabolizes ATP into AMP and then into ADO through the action of an ecto-5'nucleotidase (Magalhães-Cardoso *et al.,* 2003), which is feed-forwardly inhibited by ATP and/or ADP (Cunha *et al.,* 1996a). Interestingly, at the NMJ, AMP can be alternatively deaminated into the inactive metabolite, IMP through the action of 5'AMP-deaminase at the rat NMJ, thus bypassing ADO formation (Magalhães-Cardoso *et al.,* 2003). Moreover, ADO can either be released as such, from activated nerve terminals, Schwann cells and activated muscle fibers (reviewed in Cunha, 2005). Although there are no evidences of accumulation of ADO in synaptic vesicles or the release of this molecule as a quantum, the presence and accumulation of extracellular ADO in the synapses is related to the release of neurotransmitters and also with the frequency and intensity of neuronal firing (reviewed in Cunha, 2005). Cunha *et al.,* (1996a) and Wieraszko and Seyfried (1989) demonstrated that ATP release is greater the higher the frequency of nerve stimulation and the contribution of ADO derived from ATP increases by enhancing frequency nerve stimulation. On the other hand, the contribution of ADO released through equilibrative nucleoside transporters is predominant at lower nerve stimulation frequencies (Correia-de-Sá *et al.,* 1996; Cunha *et al.,* 1996b). In basal conditions, the intracellular concentration of ADO is typically around 10-50 nM in the cell types where it was so far quantified. When intracellular levels of ADO exceed its extracellular concentration, for example under stressful situations where the exacerbation of intracellular ATP consumption exceeds its

capacity of rephosphorylation, transport through equilibrative nucleoside transporters is reversed, i.e., there is an increase in the extracellular ADO (Geiger and Fyda, 1991). Extracellular adenosine can be inactivated by cellular uptake through the equilibrative nucleoside transporters (Geiger and Fyda, 1991) or by deamination to inosine by adenosine deaminase (ADA) (Correia-de-Sá and Ribeiro, 1996). The extracellular adenosine is able to act on metabotropic adenosine receptors located in the cell membrane of neighbouring cells (as well as of the cell that released adenosine). The activation of the different types of adenosine receptors can then modify cell metabolism according to the set-up of ADO receptors and to the primary metabolism of each particular cell type (Cunha, 2005). Although ADO does not meet all the requirements to be considered a neurotransmitter, it is able to modulate the activity of the nervous system at a presynaptic level, exerting its action through its specific receptors (Correia-de-Sá *et al.,* 1996; Cunha, 2001).

2.1. Adenosine receptors activation – P1 receptors

ADO as a neuromodulator mediates its physiological effects via cell surface receptors. ADO receptors have seven putative transmembrane (TM) domains and are coupled to heterotrimeric G proteins. There are four types of metabotropic receptors, denominated as A_1, A_{2A}, A_{2B} and A_3 receptors (Fredholm *et al.*, 2001). A_1 and A_3 receptors are coupled to Gi/o inhibitory proteins, while A_{2A} and A_{2B} are coupled to Gs excitatory proteins (Linden, 2001 and Ribeiro *et al.*, 2003). The binding of ADO to its receptor triggers a series of signal transduction mechanisms that are initiated by the receptor associated G proteins (Figure 6). ADO action depends on the receptor density, affinity and location, and in general the agonist is more efficient, when higher densities of the receptors are present. Therefore, low endogenous ADO levels, the ones observed under basal conditions, have the potential to activate the receptors only when they are in higher numbers, and not when these receptors are sparse (Fredholm *et al.,* 2001). At the rat NMJ, high-affinity A_1 and A_{2A} receptors are responsible for the major effects exerted by the ADO, namely at modulating synaptic transmission. Co-existence of both inhibitory A_1R and facilitatory $A_{2A}R$ on the same nerve terminal was first proved using neurochemical and electrophysiological methods at the rat NMJ (Correia-de-Sá *et al.,* 1991); later on it was shown that ADO could facilitate the release of neurotransmitter via activation of cAMP-coupled $A_{2A}R$ (Correia-de-Sá and Ribeiro 1994). The dual modulatory role of ADO via presynaptic inhibitory A_1R and facilitatory $A_{2A}R$ is highly dependent on the nerve stimulation pattern (Correia-de-Sá *et al.,* 1996),

particularly when this nucleoside is build-up from the catabolism of ATP release (Magalhães-Cardoso *et al.,* 2003). The tonic inhibitory effect mediated by A_1R, is observed at low frequency stimulation under resting conditions, where low amounts of ADO activate predominantly inhibitory A_1R. High-frequency, high-intensity motor nerve stimulation potentiates the tonic adenosine $A_{2A}R$-mediated facilitation of ACh release, due to accumulation of ADO in the synaptic cleft, which may overcome muscular tetanic fade, whereas activation of the inhibitory A_1R becomes less effective (Correia-de-Sá *et al.,* 1996). During 50 Hz-trains, ATP is able to reach high levels, enough to inhibit CD73. Interburst intervals, allows the recovery from CD73 enzymatic inhibition, because there is a delayed burst-like formation of ADO, leading to high synaptic concentrations of ADO, similar to those required to promote the activation of $A_{2A}R$ (Correia-de-Sá *et al.,* 1996). $A_{2A}R$ act via subtle modifications of the presynaptic inter-receptor dynamics (Sebastião and Ribeiro, 2000) involving the generation of intracellular second messengers, such as cAMP (Correia-de-Sá and Ribeiro 1994) and Ca^{2+} (Correia-de-Sá *et al.,* 2000). It worth noting that fine-tuning control of facilitatory nAChRs containing α3β2 subunits (Faria *et al.,* 2003) and muscarinic M_1 and M_2 (Oliveira *et al.,* 2002) receptors, is mediated by endogenous ADO. In parallel, there is a co-ordinate shift in Ca^{2+} cell dynamics operating ACh exocytosis, from the prevalent P/Q-type to the "facilitatory" L-type channel, in a way completely reversed by blocking $A_{2A}R$ activation (Oliveira *et al.,* 2004). These mechanisms represent a novel form of synaptic plasticity mediated by ADO and may function to overcome neuromuscular tetanic depression during neuronal firing. Neurotransmission failure in MG is particularly evident during intense motor nerve activity, a situation where ADO, acting via $A_{2A}R$, has a key role by promoting increases in the safety margin of NMT (Correia-de-Sá and Ribeiro 1996). Recently, our group demonstrated that $A_{2A}R$ fine-tuning control of NMT is impaired in animals models of MG (Noronha-Matos *et al.,* 2011; Oliveira *et al.*, 2015a). This seems to be mainly due to a decrease in endogenous ADO levels, leading to a reduction on tonic $A_{2A}R$ activity, which can be functionally recovered by application of the ADO precursor, AMP (Noronha-Matos *et al.,* 2011; Oliveira *et al.*, 2015a).

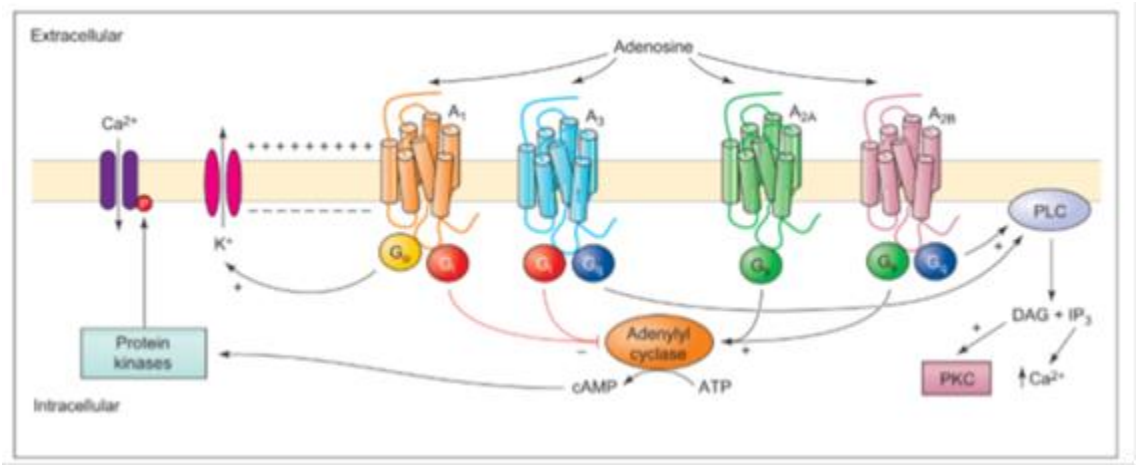

Fig. 6 - Signal transduction pathways associated with the activation of the human adenosine receptors (adapted from Moro *et al.*, 2005).

3. AIM

Recently, our group demonstrated that endogenous ADO accumulation in myasthenic motor endplates is insufficient to sustain transmitter release demand through tonic activation of presynaptic facilitatory $A_{2A}R$ (Noronha-Matos *et al.,* 2011; Oliveira *et al.,* 2015a). Considering that $A_{2A}R$ is preferentially activated by ADO originated from the catabolism of released adenine nucleotides catalysed by ecto-5'-nucleotidase/CD73 (Cunha *et al.,* 1996a) and that imparement of ADO tone regulating neurotransmitter release at the motor endplate is not associated with deficiencies in the activity of this enzyme (Oliveira *et al.,* 2015a), one may speculate about defects occurring in the release of ATP or in its extracellular metabolism by NTPDases upstream ultimate ADO formation by the ecto-5'-nucleotidase/CD73.

In this context we decided to quantify the amount of nerve-evoked ATP released from motor endplates and to evaluate the kinetics of the extracellular metabolism of ATP at phrenic nerve-hemidiaphragm preparations from EAMG animals.

As previously mentioned, motor nerve terminals are equipped with ATP-sensitive P2R in addition to P1 ADO receptors. It, thus, might happen that released ATP may undertake a role of its own to control neuromuscular transmission independently of its role as a source of ADO, but this has never been investigated in EAMG animals.

4. Materials and methods

4.1. Induction and clinical assessment of Experimental Autoimmune Myasthenia gravis (EAMG) rat models

Female rats (*Wistar Han*, 100 g) (Charles River, Barcelona, Spain) were kept at a constant temperature (21ºC) and a regular light (07.00h–19.00h)–dark (19.00h 07.00hh) cycle, with food and water *ad libitum* and randomly divided into three groups. Under general anesthesia, with ketamine (75 mg/kg) and medetomidine (100mg/kg) by intraperitoneal administration (Oliveira *et al.,* 2015a) for the EAMG model, the rats were anaesthetized and immunized subcutaneously at four sites (two hind footpads and shoulders) with 50 µg of R97-116 peptide (DGDFAIVKFTKVLLDYTGHI, JPT Peptide Technologies GmbH) – a synthetic peptide that corresponds to a specific region on the α subunit of the rat nicotinic AChR – in CFA (Complete Freund's Adjuvant) (Sigma, St. Louis, MO, USA), on day 0 and were then submitted to a second boost on day 30 with the same peptide in IFA (Incomplete Freund's Adjuvant) (Baggie *et al*., 2004; Oliveira *et al.,* 2015a). The control group was immunized with CFA and IFA emulsions, at the same time as the EAMG models, but instead of nAChR R97-116 peptide they were injected with phosphate-buffered saline (PBS). The naive group animals were not submitted to any kind of treatment. Evaluation of disease manifestations in immunized rats was performed by testing muscular weakness. Clinical scoring was based on the presence of tremor, hunched posture, general behavior, fatigability and the overall appearance of the animal. Disease severity was graded as follows: grade 0, normal strength and no fatigability; grade 1, mildly decreased activity and weak grip or cry; grade 2, clinical signs present at rest; grade 3, severe clinical signs at rest, no grip, moribund; and grade 4, dead (Baggi *et al.,* 2004). Each animal was weighed and evaluated for disease manifestation twice weekly until euthanized by decapitation (Baggi *et al.,* 2004). Animal handling and experiments were in accordance with the guidelines prepared by Committee on Care and Use of Laboratory Animal Resources (National Research Council, USA) and followed the European Communities Council Directive (86/609/ EEC).

4.2. Preparation and experimental conditions

All animals were euthanized by decapitation, using a guillotine, a fast method, which has the advantage of allowing a good exsanguination; this may be required to enable the collection of blood samples for subsequent procedures. Then, the animals were submitted to surgical isolation of the phrenic nerve hemidiaphragm as described

by Correia-de-Sá and Collaborators (1991) (Figure 7). The experiments were performed using either left or right phrenic nerve-hemidiaphragm preparations (4-6 millimeters (mm) width). Each muscle was superfused (5 mL.min^{-1}, 37°C, pH 7.4) with gassed (95% O_2; 5% CO_2) Tyrode's solution (pH 7.4) containing (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1, NaH_2PO_4 0.4, $NaHCO_3$ 11.9 and glucose 11.2, at 37ºC (Correia-de-Sá *et al.,* 1991).

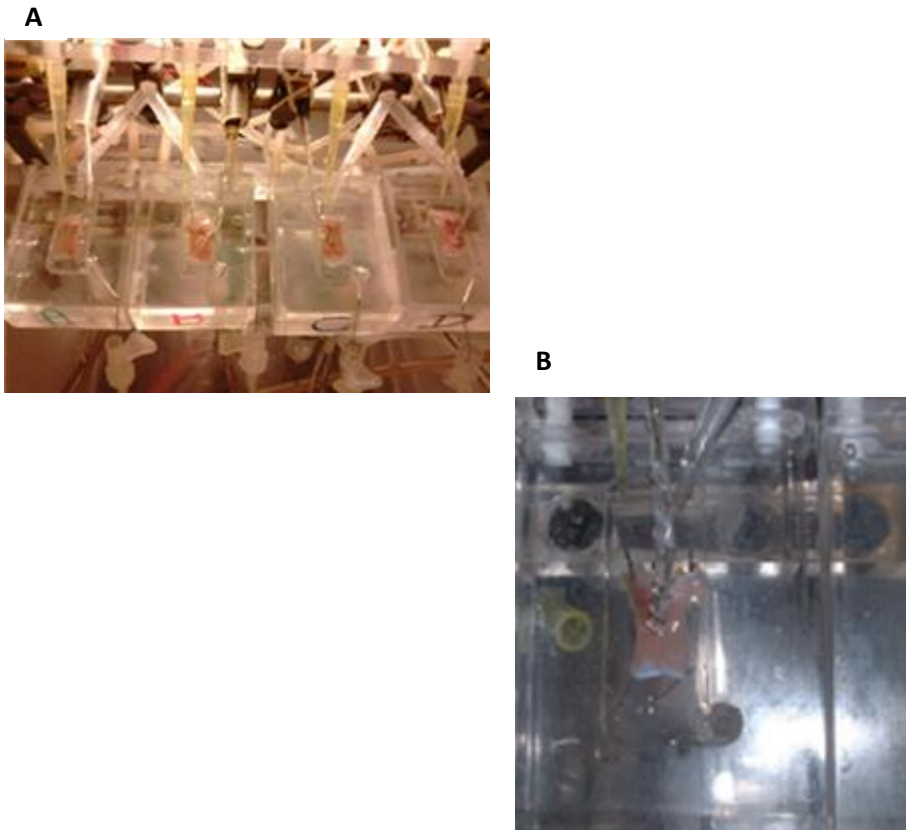

Fig. 7 - Isolated phrenic nerve-hemidiaphragm preparations mounted horizontally in thermostatized organ bathes used to quantify the release of [^{3}H]ACh and endogenous ATP. A-Preparations were mounted horizontally across the costal and the tendinous portion (phrenic center) with 4 surgical pins. B- Each phrenic nerve was inserted inside a suction electrode manufactured in the Laboratory used to promote phrenic nerve electrical stimulation.

4.3. [^{3}H]ACh release experiment from phrenic nerve hemidiaphragm preparations

The procedures used for labeling the preparations and measuring evoked [^{3}H]-acetylcholine ([^{3}H]ACh) release, have been previously described (Correia-de-Sá *et al.,* 1991). Briefly, phrenic nerve-hemidiaphragm preparations were mounted in 3-mL

capacity Perspex chambers heated to 37ºC. After a 30 min equilibration period, the perfusion was stopped and the nerve terminals were labeled for 40 min with 1µM [^{3}H]-choline (specific activity 2.5 µCi/nmol) under electrical stimulation, at a frequency of 1 Hz (0.04ms duration, 8mA). The phrenic nerve was stimulated with a glass–platinum suction electrode, placed near the first division branch of the nerve trunk, to avoid direct contact with muscle fibres (Figure 8). After the labeling period, the preparations were again superfused (37.5 mL/min) and the nerve stimulation ceased. From this point onwards, hemicholinium-3 (10 µM) was present to prevent the uptake of [^{3}H]-choline and the synthesis of unlabeled ACh. After a 60 min washout period 1.5 mL, bath samples were automatically collected every 3 min using a fraction collector (Gilson, FC 203B, France) coupled with a peristaltic pump (Gilson, Minipuls3, France) programmed device by emptying and refilling the organ bath with the solution in use. The release of [^{3}H]ACh was evoked by two periods of electrical stimulation of the phrenic nerve, 5 Hz (750 pulses), starting at min 12 (S_1) and min 39 (S_2), after the end of washout (zero time). Test drugs were added 15 min before S_2 and were present up to the end of the experiments. Medium incubation aliquots (0.4 mL) were added to 3.5 mL of Packard Insta Gel II (USA) scintillation cocktail so that tritium content samples could be measured by liquid scintillation spectrometry (counting efficiency of 40±2%). Radioactivity is expressed as DPM (disintegrations per minute). The evoked release of [^{3}H]-ACh was calculated by subtracting the basal tritium outflow from the total tritium outflow during the stimulation period (Correia-de-Sá *et al.,* 1996). The change in the ratio between the evoked [^{3}H]ACh released during the two stimulation periods (S_2/S_1), relative to the observed in control situations (in the absence of test drugs) was taken as a measure of drugs effects.

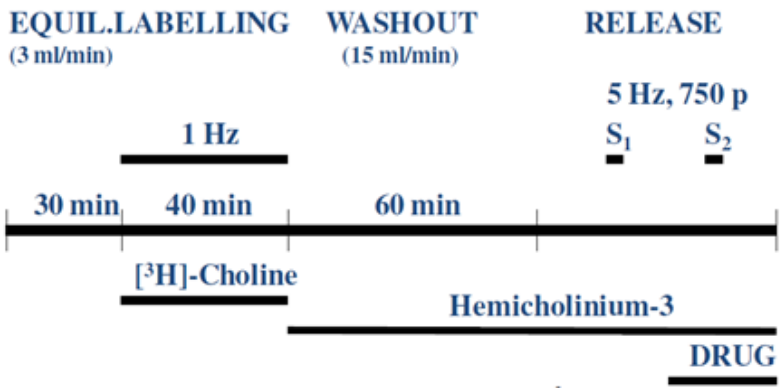

Fig. 8 - Schematic representation of the experimental procedure for [^{3}H]ACh release experiments.

4.4. Release of endogenous ATP from phrenic nerve hemidiaphragm preparations

For ATP release experiments, the innervated hemidiaphragm preparations were mounted as described previously for radiochemical experiments. After a 30 min equilibration period, the perfusion was stopped and aliquots of 1.5 mL bath samples collected automatically every 3 min. Samples were collected for 24 minutes and ATP release was induced by electrical stimulation to the time 12 with the frequency of 5Hz 750 pulses. Two hundred microliter (µL) aliquots were introduced into pre-cooled microtubes, which were frozen in liquid nitrogen until analysis The ATP content of the samples was evaluated by the luciferin – luciferase ATP bioluminescence assay kit HS II (Roche Applied Science, Indianapolis, Indiana). Luminescence was determined using a multi detection microplate reader (Synergy HT, BioTek Instruments) (Oliveira *et al.,* 2015b).

4.5. Kinetic experiments of extracellular catabolism of ATP nucleotides and nucleosides

The extracelular ATP catabolism was evaluated, at 37°C, on phrenic-nerve hemidiaphragm preparations from naïve, control, and EAMG rats. After a 30min equilibration period, the organ bath was emptied and 2mL of a 30µM ATP in gassed Tyrode's solution was added to the preparations at time zero. Samples of 75µL were collected from the bath at diferente times up to 45 min for HPLC with UV detection (HPLC-UV, LaChrome Elite, Hitachi, Merck, Germany) analysis of the variation of substrate disappearance and product formation (Magalhães-Cardoso *et al.,* 2003; Pinheiro *et al.,* 2013). In all experiments, the concentration of products at the different times of sample collection was corrected by subtracting the concentration of products in samples collected from the same preparation incubated without adding substrate. Only IMP, inosine (INO), and hypoxanthine (HX) were spontaneously released from the preparations in concentrations that did not exceed 1µM (Magalhães-Cardoso *et al.,* 2003). There was no spontaneous degradation of ATP at 37°C in the absence of the preparation. Concentration of the substrate and products were plotted as a function of time (progress curves). The following parameters were analyzed for each progress curve: half-life time ($t_{1/2}$) of the initial substrate, time of appearance of the diferente concentrations of the products, and concentration of the substrate.

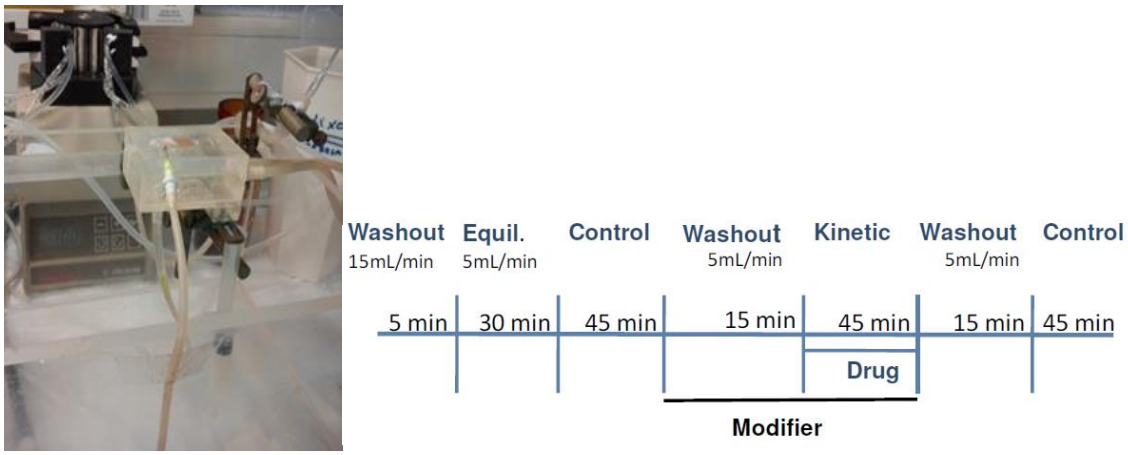

Fig. 9 - Schematic representation of the experimental procedure for the kinetic experiments.

4.5.1. Separation and quantification of ATP nucleotides and nucleosides by high-performance liquid chromatography (HPLC) analysis

Separation of ATP and their catabolism products was carried out by ion-pair reverse-phase chromatography (IP-RP-HPLC-UV) according to the method described by Cascalheira and Sebastian (1992), with minor modifications. The eluent, pH 6.0, was composed of (60mM) KH_2PO_4, (5mM) tetrabutylammonium phosphate and 5-35% (v/v) methanol. The elution program was as follows: a linear gradient from 5% to 35% (v/v) methanol during 10 minutes followed by a descendent linear gradient over a period of 8 minutes for reestablishing the initial elution conditions: absorbance values and internal pressure of the system. The mobile phase flow was 1,25 mL/min. Chromatographic identification of nucleotides and nucleosides containing samples was performed by comparison with the retention time of high purity standard, separated under the same chromatographic conditions. The chromatograms obtained after 25 µl solutions injection from standards and samples are shown in figure 10. The standards/samples were quantified through the external standard method (Cassiano *et al.,* 2009). For each component of the mixture (ATP, ADP, AMP, IMP, ADO, INO, HX) a calibration curve was prepared (graph peak area *versus* concentration) with a linear slope within the expected range of concentrations for each compound, and interception with the ordinate axis at zero or near zero. The concentration of each compound of the sample was determined through the mathematical expression of the straight lines calibration (y= mx+b), given that it is proportional to the analytical signal (area) since the injected volumes are accurately known. This method requires a strict control of technical and instrumental conditions (separation conditions, mobile phase flow,

injection volume) to obtain the calibration curves of the compounds (ATP, ADP, AMP, IMP, ADO, INO, HX) used in the adenine nucleotides/nucleosides quantification. Standard solutions of the compounds were injected (25 µL) with increasing concentrations (1.88µM – 30µM), represented in figure 11.

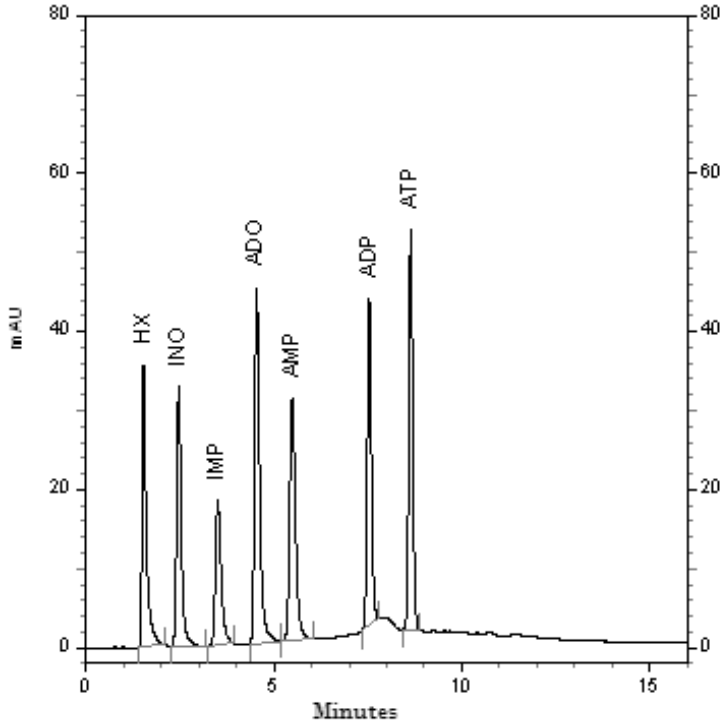

Fig. 10 - HPLC chromatogram illustrating the separation of ATP nucleotides and nucleosides in standards samples.

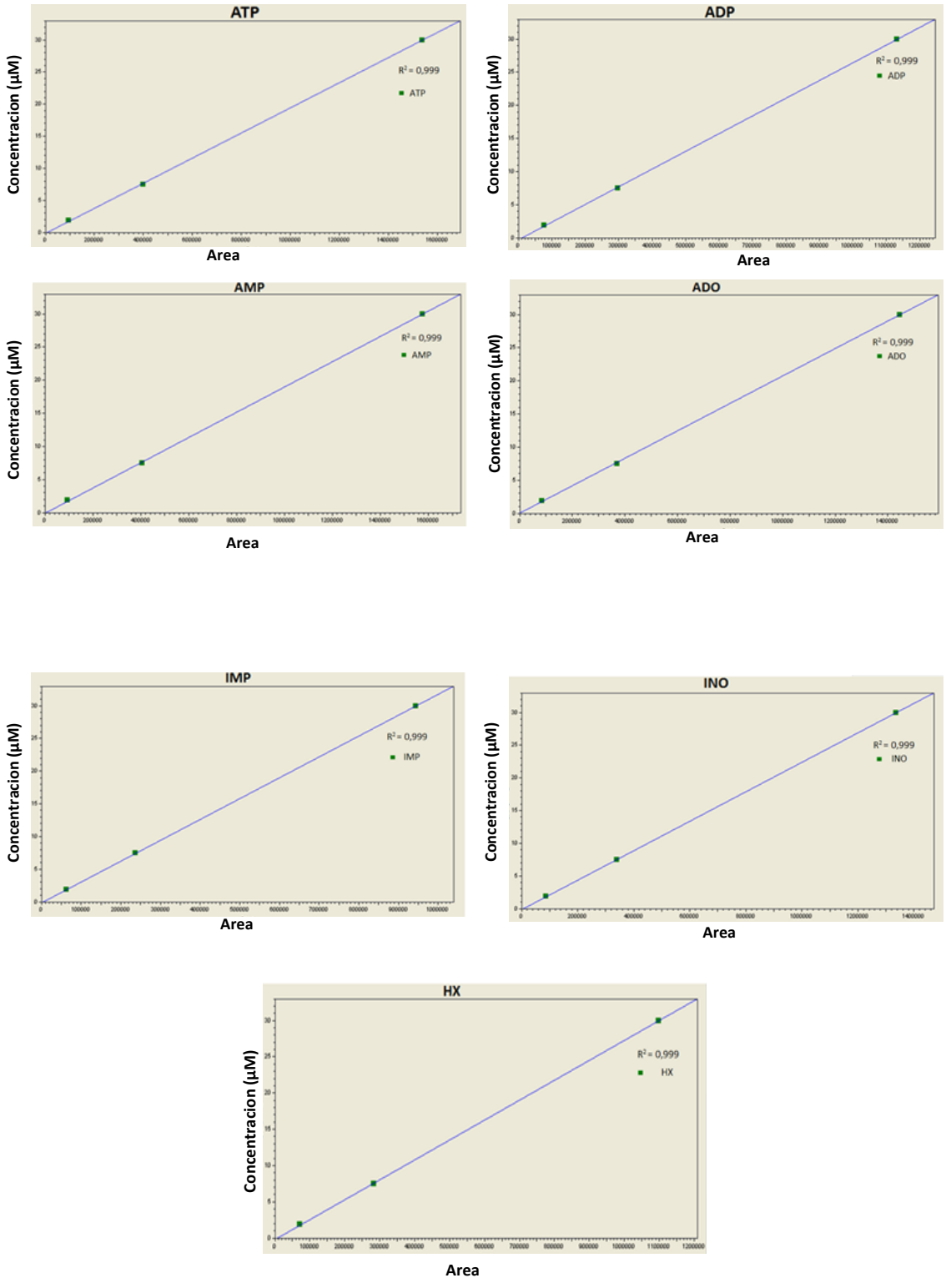

Fig. 11 - Calibration curves of ATP nucleotides and nucleosides used in this study.

4.6. Determination half-life time ($t_{1/2}$)

The half-life time ($t_{1/2}$) expresses the period of time required for the amount or concentration of the compound to decrease by one-half.

When the kinetics is a first-order kinetics, the graphical representation is linear [log (concentration) = f (time)].

The mathematical expression of the line is given by the following equation (Shargel and Yu, 1980):

$$logA = -\frac{Kt}{2,3} + logA_0$$

(A-compound concentration; $-\frac{k}{2,3}$ - slope of the straight; A_0 - y-axis interception; t - time)

The half-life time of this equation results on:

$$t_{1/2} = \frac{0,693}{K}$$

K is a constant expressed as time $^{-1}$ (k= slope of the straight × 2,3)

4.7. Drugs and Solutions

ATP; HX; INO; IMP; ADO; AMP; ADP; choline chloride; hemicholinium-3; βγimidoATP; CFA, and IFA were obtained from Sigma, St. Louis, MO, USA. The scintillation cocktail (Insta – gel Plus) were obtained from Perkin Elmer (Boston, USA); R97-116 peptide (DGDFAIVKFTKVLLDYTGHI) was obtained from JPT Peptide Technologies GmbH. All stock solutions were stored as frozen aliquots at -20ºC. Dilutions of these stock solutions were made daily.

5. Results and discussion

5.1. Endogenous release of ATP and its subsequent extracellular catabolism at the neuromuscular junction of EAMG animals

The facilitatory tonus operated by pre-synaptic adenosine $A_{2A}R$ is impaired at motor endplates of myasthenic animals (Oliveira *et al.,* 2015a). This seems to be mainly due to a decrease in endogenous ADO amounts occurring during neuronal activity, but not in resting conditions (Oliveira *et al.,* 2015a), a situation that can be fully reversed by incubation of the muscles with the ADO precursor, AMP (Noronha-Matos *et al.,* 2011; Oliveira *et al.,* 2015a). It is well established that at the rat neuromuscular junction ADO can either be released as such or can be formed from the sequential extracellular catabolism of released ATP (Cunha *et al.,* 1996a). In this context we decided to evaluate the amount of evoked ATP release from control (CFA) and EAMG animals using the luciferin-luciferase bioluminescence assay.

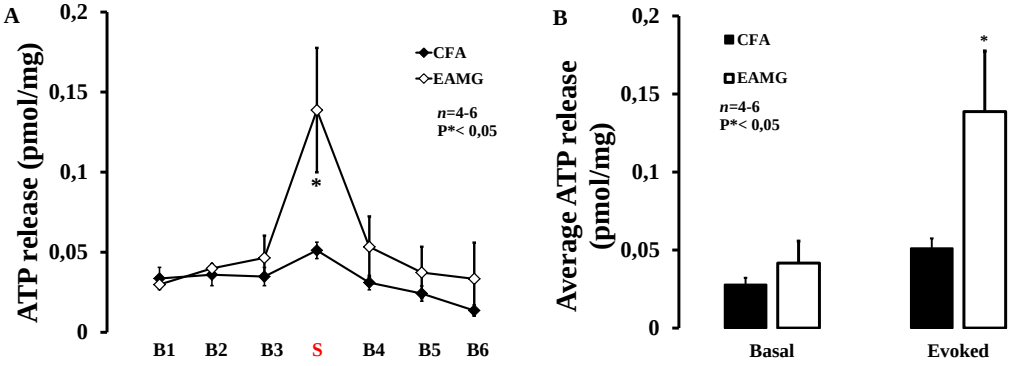

Fig. 12- A- Time course of ATP release (pmol/mg) quantified in the effluent from phrenic nerve hemidiaphragm preparations of CFA and EAMG animals by the luciferin-luciferase assay. The effluent was collected every 3 min during a period of 30 min and the phrenic nerve trunk was electrically stimulated with 750 pulses applied at 5 Hz frequency. For the sake of clarity, in the figure it is only presented the 3 points before (6', 9', 12') and after (18', 21', 24') the released period were the evoked released ATP was observed (at the period of 15 min incubation) B- Average basal and electrically induced ATP release (pmol/mg) in both CFA and EAMG animals. *P**<0,05 (Unpaired Student T' test) when comparing the average ATP release from EAMG with CFA animals.

The Fig.12A illustrates the time course of endogenous ATP release in both EAMG and CFA animals groups. Results shows that ATP release during resting conditions remained fairly constant during collection of samples and the values obtained were not significantly (P>0.05) different among the two groups, CFA (0,028±0,004 pmol/mg, *n*=6) and EAMG (0,042±0,014 pmol/mg, *n*=4) (Fig.12B). Phrenic nerve stimulation (750 pulses delivered at 5 Hz-frequency) elicited the release of ATP above basal levels into the bath effluent from both groups of animals. Interestingly, the amount of ATP

released from stimulated motor endplates from EAMG animals was significantly ($P<0.05$) higher (0,139±0,039 pmol/mg, *n*=4) than that obtained in the control (CFA) group (0,051±0,006 pmol/mg, *n*=6) (Fig.12B).

Although we did not attempted to investigate the origin of ATP released at the neuromuscular junction of EAMG animals, one might speculate that it may be influenced by the morphological changes occurring at motor endplates of these animals, which include a reduction in the total area of nAChR labeling per endplate indicating a decrease in the number of effective postsynaptic nAChR (Oliveira *et al.,* 2015a). In fact, these morphological changes reduce the safety margin of neuromuscular transmission and, thus, activation of postsynaptic nAChR, leading to a reduced skeletal muscle contractile response in EAMG animals (Oliveira *et al.,* 2015a). So, considering that the majority of the ATP release is derived from activated muscle fibers and that EAMG animals present a functional impairment on muscle fibers activation, it is plausible to hypothesize, that the observed increase in ATP release could be originated from the motor nerve terminals compartment.

On the other hand, increased ATP accumulation at the neuromuscular junction during neuronal activity may also result from changes in its extracellular metabolism, which in addition to surplus ATP release may increase the quantified levels of the nucleotide. In fact, EAMG animals exhibit higher amounts of ATP released from stimulated preparations (Fig.12) and the amounts of evoked ADO release are significantly reduced (Oliveira *et al.,* 2015a). So, it is plausible that deficits in extracellular metabolism of ATP may operate in myasthenic animals. Figure 13 illustrates the time course of the extracellular catabolism of ATP and the formation of its metabolites on phrenic nerve-hemidiaphragm preparations of Naïve and EAMG rats. The ATP metabolites detected in the bath were ADP (Fig. 13B), AMP (Fig.13C), adenosine (ADO) (Fig.13D), inosine (INO) (Fig.13F) and hypoxanthine (HX) (data not shown), whose concentrations increased with time. The kinetics of the extracellular catabolism of ATP (30 µM) in EAMG rats was roughly similar to that of Naïve animals (Fig. 13A). ATP (30 µM) was metabolized with a half-degradation time of 8±2 min (*n*=8) in Naïve rats (Magalhães-Cardoso *et al.,* 2003) and of 5±1 min (*n*=4) in EAMG animals (this study). Coincidence or not, we observed an increased accumulation of ADP in the bath effluent during the first 15 min after addition of the substrate in EAMG rats compared to Naïve animals (Fig. 13B).

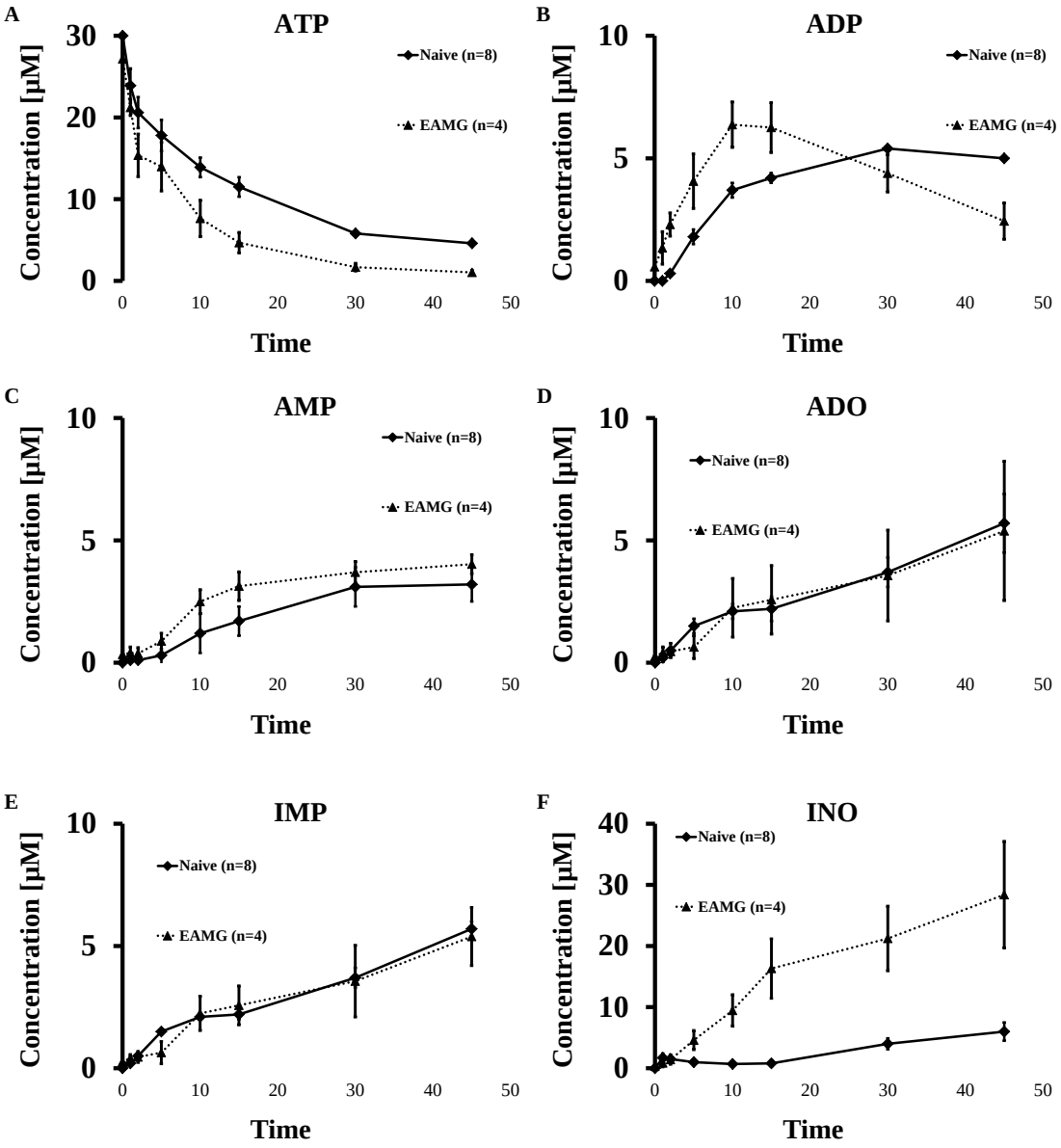

Fig. 13- Time course of extracellular ATP catabolism in phrenic nerve hemidiaphragm preparations from Naïve and EAMG animals. ATP (30µM) was added at zero time to the preparation and samples were collected from the bath at the times indicated on the abscissa and retained for HPLC analysis. (A), (B), (C), (D), (E) and (F) show the kinetics of the extracellular ATP, ADP, AMP, ADO, IMP and INO, respectively. Shown is pooled data from a number of experiments (shown in parentheses). The vertical bars represent SEM and are shown when they exceed the symbols in size.

No changes were observed in the rate of AMP (Fig. 13C), IMP (Fig. 13E) and ADO (Fig. 13D) formation in the two groups of animals.

Among the plasma membrane bound NTPDases (NTPDases 1, 2, 3 and 8), NTPDase1 (also named CD39, ATPDase, ecto- apyrase, ecto-ADPase) hydrolyzes ATP and ADP equally well, NTPDase2 is a preferential triphosphonucleosidase leading to transient

ADP accumulation, whereas NTPDase3 (CD39L3, HB6) and NTPDase8 are functional intermediates between NTPDases 1 and 2 (see Zimmermann, 2001). Thus, increased ADP accumulation from the extracellular ATP catabolism implicates a significant NTPDase 2 activity in EAMG animals. Confirmation of the NTPDase enzyme subtype most expressed in myasthenic rats needs further investigations.

Overall, data presented so far indicate that deficits in the extracellular ATP metabolism by ectonucleotidases (NTPDases and ecto-5'-nucleotidase) are not responsible for the accumulation of the nucleotide in the incubation fluid during neuronal activity observed in EAMG animals. ATP can be released to the extracellular compartment by non-lytic mechanisms including: (1) exocytosis of ATP-containing vesicles; (2) through nucleotide-permeable channels (connexin and pannexin hemichannels, maxi-anion channels, volume regulated anion channels or P2X7 receptor channels); (3) via transport vesicles that deliver proteins to the cell membrane; (4) via lysosomes (Dahl and Muller, 2014; Penuela *et al.,* 2013). One of these mechanisms can potentially be operating to increase evoked ATP release at motor endplates of EAMG animals. So, understanding which mechanisms underlies the increased release of evoked ATP in myasthenic conditions needs further investigation.

Considering that ATP is able to modulate neuromuscular transmission through the activation of P2R (Salgado *et al.,* 2000; Voss, 2009) or P1R through its conversion into ADO (Cunha *et al.,* 1996a), it will be interesting to understand the dynamics of ATP neuromodulation of neuromuscular transmission.

5.2. Neuromodulatory role of ATP on neuromuscular transmission in healthy and EAMG animals

Exogenously applied ATP caused a biphasic effect on [^{3}H]ACh release induced by phrenic nerve stimulation with 750 pulses applied with a 5Hz frequency (Fig.14A). Pre-treatment of phrenic nerve hemidiaphragm preparations during 15 min with ATP (0.3-30 µM) significantly increased evoked [^{3}H]ACh release, in which the maximal facilitatory effect (56±7%, *n*=5) was observed when ATP was applied at 1 µM concentration. However, the increase of ATP concentration to 100 µM revealed an inhibitory effect on evoked [^{3}H]ACh release (39±6%, *n*=7).

At the rat motor nerve terminals ATP is converted into ADO which activates P1R (Cunha *et al.,* 1996a). In order to assess the ATP effect on neuromuscular transmission without the interference of the ATP-ADO-P1R pathway, the dose-response curve of ATP was repeated in the presence adenosine deaminase (ADA, 0,5 U/mL), the enzyme that inactivates the ADO into its inactive metabolite (Correia-de-Sá *et al.,* 1996).

Inactivation of ADO originated from the extracellular ATP catabolism with ADA (0,5 U/mL) converted the facilitatory action of ATP (1 μM, 56±7%, *n*=5) into an inhibitory effect of (32±18%, *n*=4), suggesting that in absence of ADO a P2R action operated by ATP itself emerges (Fig. 14A).

To further confirm this hypothesis we assessed the effect of a slowly hydrolysable analogue of ATP, βγImidoATP, on evoked [^{3}H]ACh release. βγImidoATP (30 and 100 μM) concentration-dependently decreased [^{3}H]ACh release from stimulated phrenic nerve endings of CTRL rats.

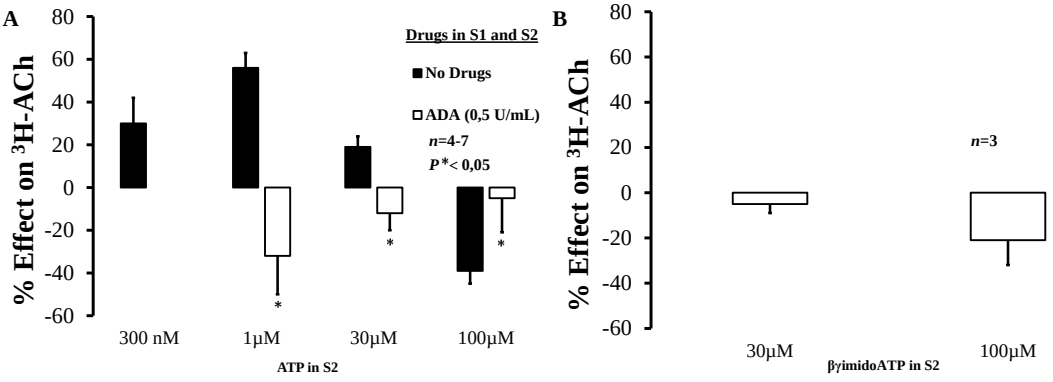

Fig. 14- A- Concentration-response curve of ATP on electrically evoked (5 Hz, 750 pulses) [^{3}H]ACh release from Naïve animals either in presence or absence of adenosine deaminase (ADA). ATP (0,3-100 μM) was applied 15 minutes before S2 and ADA (0,5 U/mL) was applied 15 minutes before S1 and S2. Ordinates represent the percentage of effect of the nucleotide by comparing the S2/S1 ratios with the S2/S1 ration in absence or in the presence of ADA. Each point is the mean±SEM of 4 to 7 experiments. **P*<0,05 (Student's T-test). B- Effect of the stable βγimidoATP on electrically evoked (5 Hz, 750 pulses) [^{3}H]ACh release from Naïve animals. βγimidoATP (30-100 μM) was applied 15 minutes before S2. Ordinates represent the percentage of effect of the βγimidoATP by comparing the S2/S1 ratios with the S2/S1 ration in absence on drugs. Each point is the mean±SEM of 3 experiments. **P*<0,05 (Student's T-test).

Moreover, if one shortens the incubation time with ATP (1 μM) from 15 to 3 min before stimulus aplication in order to decrease the time available for ATP to be

dephosphorylated into adenosine, the facilitatory effect (39±13%, *n*=7) no longer appeared and, instead, an inhibitory action (30±4%, *n*=7) emerges (Fig. 16).

These results suggest that ATP modulates *per se* neuromuscular transmission by activating inhibitory P2R and, after being converted into adenosine, acts on P1R (probably A_1R) to decrease evoked ACh release. It has been demonstrated that ATP exerts an inhibitory effect on both evoked (Sokolova *et al.,* 2003) and spontaneous (De Lorenzo *et al.,* 2006) ACh release from mouse motor nerve terminals via the activation of P2YR. Inhibitory couling to adenylate cyclase via $G_{i/o}$ proteins are most probably involved in ATP-mediated inhibition of ACh release at the neuromuscular junction. ATP-sensitive $P2Y_{12}$ and $P2Y_{13}$ belong to the group of P2YR that preferentially couple to inhibitory $G_{i/o}$ proteins (Burnstock, 2007). De Lorenzo and collaborators (2006) demonstrated that the pertussis toxin and N-etylmaleimide abolished the effect of βγ-imido ATP suggesting that the P2YR involved in the presynaptic inhibition could be coupled to $G_{i/o}$ protein. Despite, no pharmacological screening has been performed so far to characterize the subtype of P2YR at the mammalian neuromuscular junction, on may assume based on previous studies (De Lorenzo *et al.,* 2006) that either $P2Y_{12}R$ or $P2Y_{13}R$ are the most probable receptors involved.

Nevertheless, it should be noted that the predominant effect of exogenously applied ATP at 1 μM concentration is mediated by its metabolite ADO acting on facilitatory $A_{2A}R$. The effect of ATP-sensitive P2YR on neuromuscular transmission is only observed under circumstances were no adenosine is available and/or $A_{2A}R$ activation is inoperant. These include (1) the lack of ADO formation (Fig. 14A), (2) in the presence of a slowly hydrolysable ATP analogues (Fig.14B), and (3) when the exposure time is not enough to allow the conversion of ATP into ADO (Fig.16). Prevalence of the facilitatory action of $A_{2A}R$ over P2R-mediated inhibition of evoked transmitter release from motor nerve endings implicates that the ATP inhibitory response must be shut-down upon activation of $A_{2A}R$ by adenosine formed from the extracellular catabolism of the nucleotide. Previous results from our group demonstrated that adenosine $A_{2A}R$ on phrenic motor nerve terminals is positively coupled to Gs protein leading to activation of the AC/AMPC/PKA pathway (Correia-de-Sá and Ribeiro, 1994). On the other hand $P2Y_{12}R$ and $P2Y_{13}R$ are negatively coupled to Gi/o protein (Burnstock, 2007). Thus, it is likely that adenylyl cyclase is key enzyme operating the crosstalk between inhibitory P2YR and excitatory $A_{2A}R$ pathways leading to the prevalent facilitatory action of ATP on ACh release. Whether this crosstalk is perturbed in conditions where activation of

A_{2A} receptors are impaired (e.g. myasthenia gravis) (Oliveira *et al.,* 2015a), deserves to be investigated.

To this end, we tested the effect of ATP (1 µM, applied during 15 min) either in presence or absence of ADA (0.5 U/mL) on evoked [^{3}H]ACh release from phrenic nerve terminals of EAMG rats. Pre-treatment with ADA (0,5 U/mL) converted the facilitatory action of ATP (1 µM) (18±8%, *n*=5) into an inhibitory effect of (26±10%, *n*=4) in CFA animals. In EAMG rats, ATP (1 µM) applied 15 min prior stimulus delivery to the phrenic nerve facilitated [^{3}H]ACh release by (43±12%, *n*=5) in the absence of ADA (0,5 U/mL), but it failed to affect transmitter release when ADO was inactivated by ADA (0,5 U/mL, 5±17%, *n*=4). We have previously reported that A_{2A}R tonic activity is impaired in EAMG animals and it could be recovered by AMP application (Oliveira *et al.,* 2015a). So, it was not surprising that ATP application rescued the facilitatory effect of A_{2A}R activation.

Interestingly, using a shorter incubation time (3 min) with ATP (1 µM), which decreases the conversion of ATP into ADP and AMP, and subsequently to ADO via de ectonucleotidase cascade in EAMG rats, reestablished the inhibitory effect (41±3%, *n*=2) obtained in both Naïve (30±4%, *n*=7) and CFA (26±10%, *n*=4) animals, under the same experimental conditions.

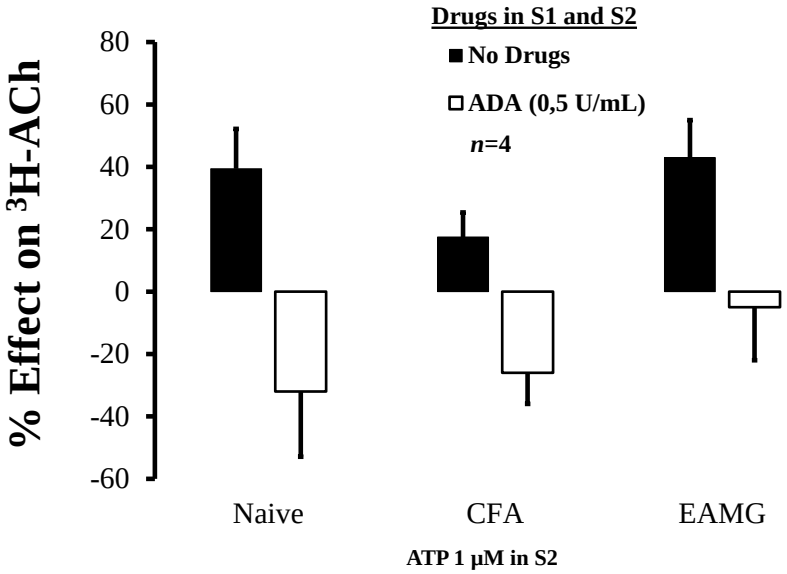

Fig. 15- Concentration-response curve of ATP on electrically evoked (5 Hz, 750 pulses) [^{3}H]ACh release from Naïve, CFA and EAMG animals either in presence or absence of adenosine deaminase (ADA). ATP (1 µM) was applied 15 minutes before S2 and ADA (0,5 U/mL) was applied 15 minutes before S1 and S2. Ordinates represent the percentage of effect of the nucleotide by comparing the S2/S1 ratios with the S2/S1 ration in absence or in the presence of ADA. Each point is the mean±SEM of 4 experiments. **P*<0,05 (Student's T-test).

Recently, it was reported that long exposition to ADP nucleotide triggers desensitization of $P2Y_{12}R$ (Hardy *et al.,* 2005; Yu *et al.,* 2014). In fact, we observed that during the first 15 minutes of ATP metabolism in EAMG animals there was a preferential ADP accumulation compared to Naïve rats (Fig.13B). Whether transient ADP accumulation, but not instantaneous ATP availability, in the extracellular milieu is sufficient to cause desensitization of P2YR in myasthenic neuromuscular junctions requires further investigations.

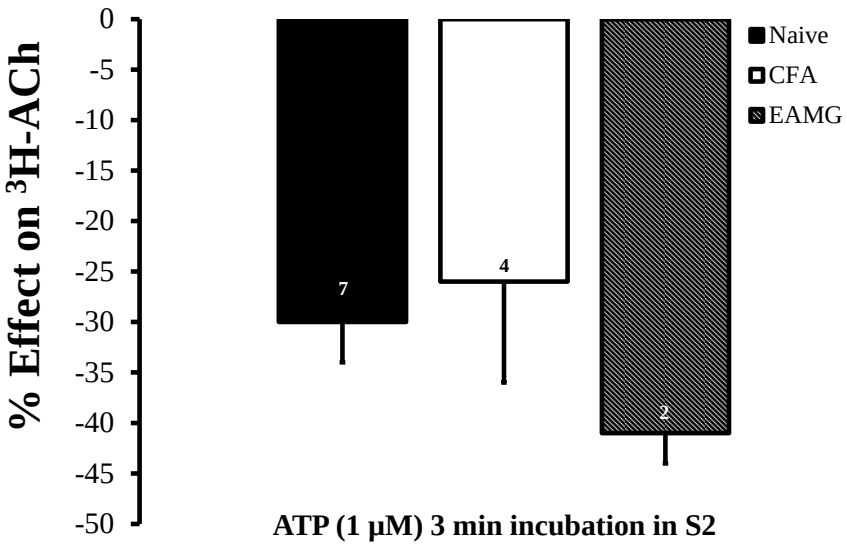

Fig. 16- Concentration-response curve of ATP on electrically evoked (5 Hz, 750 pulses) [^{3}H]ACh release from Naïve, CFA and EAMG animals. ATP (1 µM) was applied 3 minutes before S2. Ordinates represent the percentage of effect of the nucleotide by comparing the S2/S1 ratios with the S2/S1 ration in absence or in the presence of ATP. Each point is the mean±SEM of 7, 4 and 2 experiments. **P*<0,05 (Student's T-test).

6. Conclusions and future work

The major goal of this work was to provide the basis for better understanding the biochemical and molecular pathways concerning role of ATP on neuromuscular transmission in myasthenic animals.

At the rat neuromuscular junction, adenosine inhibitory A_1/excitatory A_2A receptor activation balance is dependent on the stimulation pattern (Correia-de-Sá *et al.,* 1996), which also tightly regulates the amount of extracellular adenosine build-up from the catabolism of released ATP (Cunha *et al.,* 1996a; Magalhães-Cardoso *et al.,* 2003). Previous reports from our group suggest that adenosine facilitation of ACh release operated by $A_{2A}R$ activation is impaired in individuals with myasthenia, a situation that can be rescued by incubation with ADO precursors, AMP (Noronha-Matos *et al.,* 2011; Oliveira *et al.,* 2015a) and ATP (this study). Released ATP modulates neuromuscular transmission either by directly activating P2 purinoceptors (P2R) (Salgado *et al.,* 2000) or indirectly through the activation of P1 receptors after being metabolized into adenosine (ADO), via ecto-nucleotidases (Cunha *et al.,* 1996a; Magalhães-Cardoso *et al.,* 2003). This study show that lower endogenous adenosine amounts at myasthenic neuromusuclar junctions are not owe to significant changes in the kinetics of the extracellular metabolism of adenine nucleotides, nor to decreases in the release of ATP from stimulated preparations. The ultimate cause for deficits in adenosine to reach enough levels required to activate facilitatory A_{2A} receptors may be excessive inactivation of the nucleoside by deamination and/or cellular uptake (Correia-de-Sá and Ribeiro, 1996).

The signaling via extracellular nucleotides has been recognized for over a decade as one of the most ubiquitous intercellular signaling mechanisms (Lüthje, 1989; Burnstock *et al.,* 2004). The inhibitory effects of ATP *per se* on ACh release may be mediated by P2Y receptors. From all P2YR subtypes, the most probably involved in the inhibitory action of ATP on neuromuscular transmission are the $P2Y_{12}R$ and $P2Y_{13}R$, as these receptors couple negatively to adenylate cyclase via G(i/o) proteins (De Lorenzo *et al.,* 2006) in contrast to all other subtypes that either activate PLC or AC via Gq/11 and Gs proteins leading to increases of intracellular Ca^{2+} and transmitter release facilitation.

Predominance of $A_{2A}R$ facilitation of transmitter release over P2YR-mediated inhibition in the presence of ATP may imply a negative crosstalk between these two purinoceptors, which deserves investigation in the near future. Whether this involves

homo- or hetero-desensitization of the P2YR requires further studies. Whatever the mechanism involved in this crosstalk, it might implicate the interplay at the adenylate cyclase activation level.

Another issue that might be interesting investigating in the near future is the relationship between morphological changes of myasthenic endplates and their implications on the distribution of ATP release sites, nucleotidases and purinoceptors (purinome) at the perturbed synaptic microenvironment.

Therefore, a detailed pharmacological characterization of inhibitory P2 receptors in myasthenic motor endplates becomes crucial to fully understand the pathophysiological consequences of the disease and to open the way for the development of new therapeutic strategies. This hypothesis certainly deserves further studies in the near future.

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